10,10-dimethyl-9-anthrone (**14**) and 9,10-dihydro-10,10-dimethylanthracene (**13**), as determined by the relative areas of the peaks at δ 1.71 (**14**) and 1.56 (**13**). Comparison with the area of the methylene peak at δ 4.03 (**13**) confirmed the ratio.

A second run was carried out in a similar manner, except that the reaction time was extended to 24 h. The results were identical.

(b) In Acetic Acid–Acetic Anhydride. The reaction was carried out as described above, except that a mixture of 10 mL of acetic acid and 10 mL of acetic anhydride was employed as solvent. Workup was carried out as described above except that the washing with sodium bicarbonate was omitted. NMR analysis of the product showed a **13**:**14** ratio of 3:2.

(c) In Acetic Anhydride. The reaction was carried out as described above employing 0.19 g of **17** in 20 mL of acetic anhydride. The solution (after 2 h reaction time) was diluted with water and heated on a steam bath for 10 min, then extracted with methylene chloride; the methylene chloride solution was washed with water and extracted with sodium hydroxide solution. The neutral layer was washed with water, dried over magnesium sulfate, and filtered; the solvent was evaporated to give 0.10 g of yellow oil, which NMR analysis showed to consist of **13** and **14** in a ratio of 3:2. The sodium hydroxide layer was acidified with dilute hydrochloric acid and extracted with methylene chloride; the organic layer was worked up as described above to give 93 mg of acid **12**.

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Registry No. 1, 80716-28-5; **3**, 80716-32-1; **5**, 84851-99-0; **9**, 80716-33-2; **10**, 5738-26-1; **11**, 80716-34-3; **12**, 80716-37-6; **13**, 42332-94-5; **14**, 5447-86-9; **17**, 18792-73-9; methyllithium, 917-54-4; anthracene, 120-12-7; 2-(2-methylbenzyl)benzoic acid, 80716-36-5; 9-methylanthracene, 779-02-2.

Synthesis of a Cyclic Analogue of Oxidized Glutathione by an Intersite Reaction in a Swollen Polymer Network

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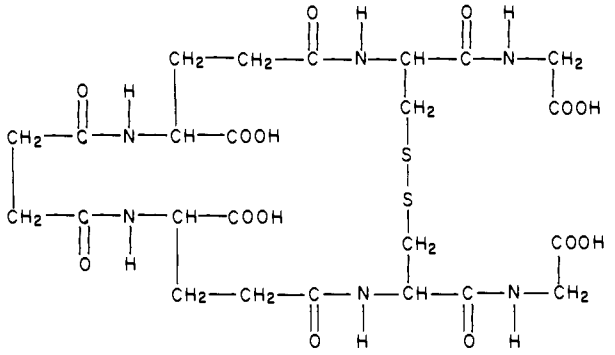
Abstract: Protected glutathione was synthesized on a 1% cross-linked copoly(styrene–divinylbenzene) resin support. Following deprotection of the α-amino groups, the chains were cross-linked in two steps. Half were acylated with succinic anhydride, with liberation of an equivalent number of carboxyl groups, which were then activated and coupled with the remaining half of the chains that still contained amines. Less than 0.5% (0.0005 mmol/g) of all the chains remained non-cross-linked. The resulting hexapeptide derivative, succinylbis[glutathione], was cleaved from the resin in HF and oxidized in air to the cyclic disulfide. The purified product was shown to be homogeneous by several chromatographic and analytical methods and to be indistinguishable from a sample prepared by solution methods. The synthesis depended on the ability to achieve a high yield of intersite reaction within the same resin bead, which required extensive flexibility of the solvent-swollen polymer matrix.

A cyclic analogue, I, of oxidized glutathione (GSSG) with



I

restricted conformation has been synthesized by solid-phase methods[1] for the purpose of studying the mechanism of action of the enzyme glutathione reductase.

This work has also provided an opportunity to examine certain aspects of the nature of the solid support used in the synthesis and in particular to answer the question of whether or not quantitative reaction between all the functional sites on the resin can be achieved. In the early period of resin-supported synthesis it was often assumed that functional sites on low-cross-linked polystyrene–divinylbenzene copolymer beads were isolated and that their reactions were analogous to reactions in solution at high dilution.[2-6] It was demonstrated, for example, that intramolecular cyclization reactions could more favorably compete with intermolecular (intersite)[7] polymerization reactions than during the same reactions carried out in solution at similar concentrations.[3] In special cases there has been evidence for long-time site isolation.[8-10] It has become more and more clear, however, that site isolation in such systems is usually a kinetic phenomenon and that site–site interaction can readily occur.[11-20] The distribution of

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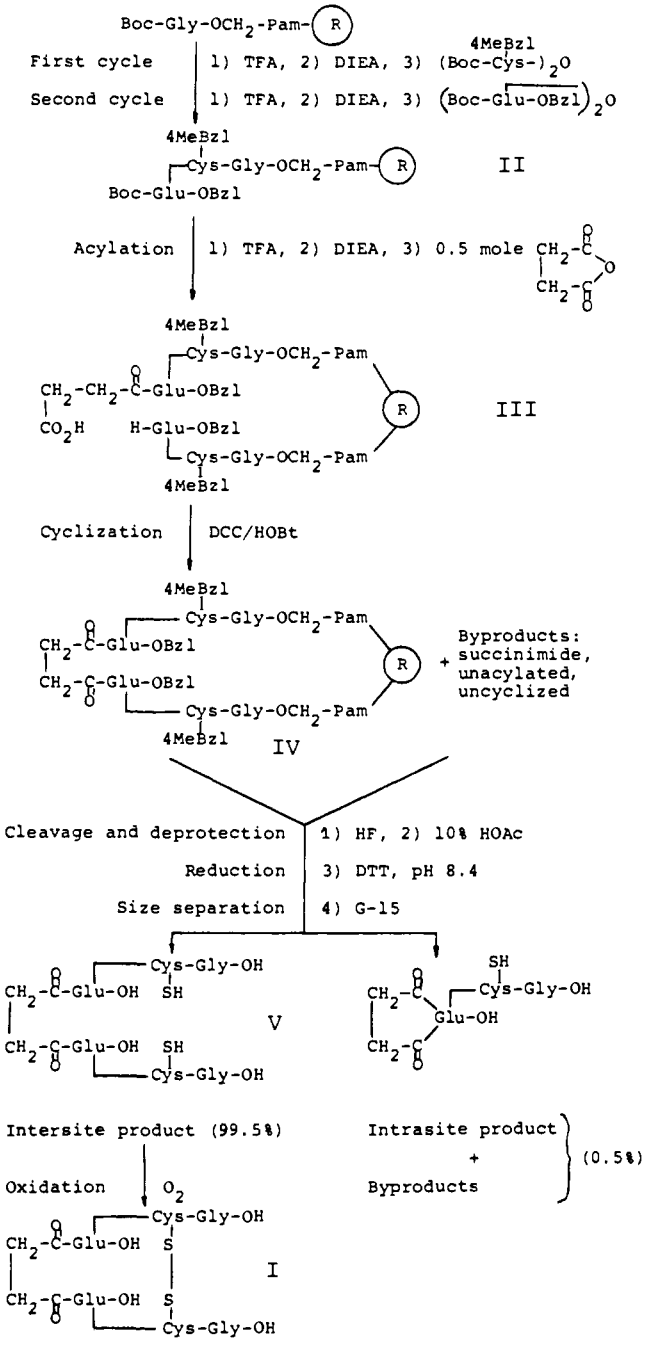
Scheme I



products depends on the relative rates of the competing reactions, on the rates of diffusion of solutes, and on the mobility of the polymer chains. In the presence of very rapid intramolecular reactions or reactions with soluble reagents very little site–site interaction occurs, but with slower reactions or in the absence of competing reactions, the site–site reaction dominates. We have

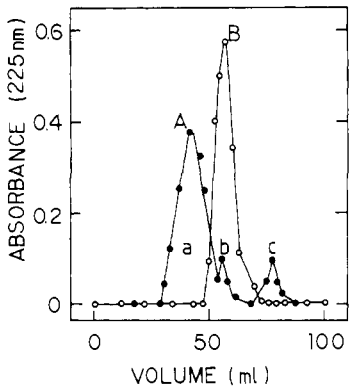


Figure 1. Chromatographic separation of succinylbis[glutathione] from synthetic byproducts on a Sephadex G-15 column (1.8 × 40 cm): curve A (●—●), crude reaction product after reduction with dithiothreitol; curve B (○—○), product after air oxidation to the cyclic hexapeptide I (elution with water at 7.3 mL/h). The product in each UV-absorbing peak was also quantitated by amino acid analysis of acid hydrolysates; on this basis peak c was only 0.5% of the total of the combined hexapeptide fractions, a + b.

previously demonstrated[21] that during solid-phase peptide synthesis the peptide chains and the lightly cross-linked polystyrene backbone exert a complementary solubilizing effect on each other, and in a suitable solvent such as dichloromethane or dimethylformamide, the peptide chains in the swollen polystyrene bead are highly solvated and react rapidly and completely with soluble acylating reagents. In the present experiments it is shown that intersite reactions between different peptide chains in the same polymer bead can proceed very nearly to completion.

The cyclic analogue I of GSSG was synthesized on an aminomethylcopoly(styrene–1% divinylbenzene) resin support[22] (Scheme I). Because of the design of the synthesis, the desired peptide could be obtained only by site–site interaction between peptide chains on the same resin bead. The system described here has the advantage that the reaction between peptide chains can be monitored to completion by using a quantitative ninhydrin test.[23] The quantitation of the desired cyclic peptide formed in this scheme is a good representation of the extent of intersite reaction possible during solid-phase peptide synthesis. As shown in the purification step, Scheme I, the tripeptide byproducts formed because of *intra*site reactions (i.e., reaction within the same peptide chain) or resulting from side reactions or incomplete reactions can be distinguished and quantitated. The tripeptide-resin II was synthesized from Boc-glycyl-4-(oxymethyl)phenylacetamidomethyl-resin (Boc-Gly-OCH_2-Pam-Res)[24,25] by normal stepwise solid-phase peptide synthesis[1] using the preformed symmetric anhydrides of the Boc-amino acids[25] (Scheme I). An initial substitution of 0.18 mmol/g styrene was selected for this study. The N^{α}-protecting group was removed by treatment with 50% trifluoroacetic acid in CH_2Cl_2. After neutralization with 5% diisopropylethylamine in CH_2Cl_2, the peptide was reacted with exactly 0.5 molar equiv of succinic anhydride in DMF.[26] The carboxyl group so generated on one-half of the peptide chains in the polymer was then coupled to the N^{α}-amino group on the remaining unreacted peptide chains, by using a dicyclohexylcarbodiimide/*N*-hydroxybenzotriazole coupling protocol in DMF. The coupling was continued until the peptide-resin showed a

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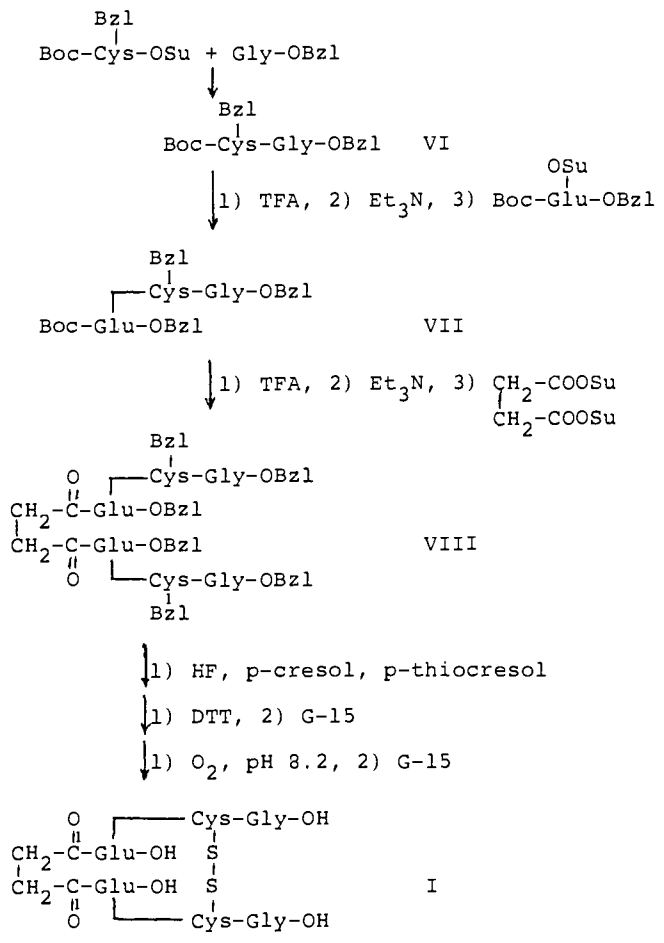
Scheme II



negative (<0.0003 mmol of NH_2/g) ninhydrin test.[27] The peptide was liberated by treating the peptide-resin with HF[28] and was extracted into 10% aqueous HOAc. The peptide mixture was reduced by treatment at pH 8.5 with dithiothreitol and was fractionated on Sephadex G-15 (Figure 1, curve A). Peptide under peaks a and b (99.5% of total by amino acid analysis) resulted from intersite reaction between two tripeptide chains within the same swollen polymer bead. After the cleavage and reduction steps, peak a contained the succinyl linkage between two reduced glutathione chains. Peak b contained a small amount of oxidized, cyclic disulfide product, which re-formed after the reduction step. Peak c contained some UV-absorbing material derived from the scavengers and a very small amount of a mixture of the possible tripeptide derivatives, e.g., unreacted GSH (established by a standard chromatogram), succinyl-GSH, the acylurea, and the succinimide formed by intramolecular cyclization of the activated succinyl peptide.

The reduced succinyl hexapeptide (peak a) was then oxidized by air in 0.1 M NH_4OAc buffer, pH 8.2 (negative Ellman's test).[29] The oxidized cyclic peptide was lyophilized and again passed through a G15 column (Figure 1, curve B). A single peak was observed, corresponding to the oxidized cyclic hexapeptide I. Peptide I was also made by classical solution chemistry according to Scheme II. The peptides obtained by the two procedures were found to be homogeneous and identical by gel filtration on Sephadex, by TLC on silica gel plates using three different solvent systems, and by ion-exchange chromatography on DEAE-Sephacel.

Experimental Section

Materials. Methylene chloride was distilled over sodium carbonate; diisopropylethylamine was distilled over ninhydrin and then over calcium hydride. Spectroscopic grade DMF (MCB) was stored over 4-Å molecular sieves. Trifluoroacetic acid was purchased from Halocarbon Products Inc. Glycine benzyl ester *p*-tosylate, Boc-L-glutamic acid α-benzyl ester, and Boc-*S*-benzyl-L-cysteine *N*-hydroxysuccinimide ester were from Vega Biochemicals. Other protected amino acids were purchased from Peninsula Laboratories. Other reagents were 4-(bromomethyl)phenylacetic acid phenacyl ester (RSA Corp., Ardsley, NY), dicyclohexylcarbodiimide, and *N*-hydroxybenzotriazole (Aldrich Chemical Co.). Polystyrene–1% divinylbenzene cross-linked resin (Bio-Beads S-X1, 200–400 mesh) was purchased (Bio-Rad Labs) and washed according to the published procedure.[30]

Aminomethyl-resin containing 0.25% N (0.18 mmol/g) was prepared according to the procedure described earlier.[22] Boc-glycyl-4-(oxymethyl)phenylacetic acid was prepared as before[24] and was isolated as the free acid after recrystallization from ethyl acetate/petroleum ether: 54% yield; mp 116–118 °C (lit.[24] mp 116–118 °C).

This derivative was coupled to the aminomethyl-resin by activation with DCC,[14] yielding Boc-glycyl-4-(oxymethyl)phenylacetamidomethyl-resin (Boc-Gly-OCH_2-Pam-Res).

Synthesis of Peptide-Resin II. The fully protected tripeptide resin was synthesized according to the protocol described below. Boc-Gly-OCH_2-Pam-Res (4 g, 0.72 mmol Gly) was placed in a reaction vessel and then treated with shaking with the following reagents in the order shown below: (1) CH_2Cl_2, 80 mL (6 × 1 min); (2) 50% TFA/CH_2Cl_2, 80 mL (1 × 1 min); (3) 50% TFA/CH_2Cl_2, 80 mL (1 × 20 min); (4) CH_2Cl_2, 80 mL (6 × 1 min); (5) 5% DIEA/CH_2Cl_2, 80 mL (2 × 2 min); (6) CH_2Cl_2, 80 mL (3 × 1 min); (7) 4 equiv of preformed (Boc-AA)$_2$O, 0.05 M in CH_2Cl_2, 2 h; (8) CH_2Cl_2, 80 mL (6 × 1 min); (9) 5% DIEA/CH_2Cl_2, 80 mL (1 × 2 min); (10) CH_2Cl_2, 80 mL (3 × 1 min); (11) 4 equiv of HOBt in 15 mL of DMF, 0 °C, add 4 equiv of DCC in 5 mL of CH_2Cl_2, stir for 10 min at 0 °C, add 4 equiv of Boc-AA-OH in 5 mL of DMF, stir for 10 min at 0 °C, transfer to reaction vessel, rinse with 25 mL of DMF, shake for 2 h at room temp; (12) CH_2Cl_2, 80 mL (6 × 1 min). About 5 mg of peptide-resin was analyzed for uncoupled amino group by quantitative ninhydrin reaction.[23] If needed, steps 8–12 were repeated.

Preparation of Peptide-Resin III. Peptide-resin II (800 mg, 0.14 mmol) was transferred to a new reaction vessel and treated as follows: (1) CH_2Cl_2, 15 mL (6 × 1 min); (2) 50% TFA/CH_2Cl_2, 15 mL (1 × 1 min); (3) 50% TFA/CH_2Cl_2, 15 mL (1 × 20 min); (4) CH_2Cl_2, 15 mL (6 × 1 min); (5) 5% DIEA/CH_2Cl_2, 15 mL (2 × 2 min); (6) CH_2Cl_2, 15 mL (3 × 1 min); (7) 0.070 mmol of succinic anhydride in 7 mL of DMF, 24 h; (8) DMF, 15 mL (6 × 1 min); (9) CH_2Cl_2, 15 mL (6 × 1 min); (10) 3.5 mg of the resin was dried on a vacuum pump and then analyzed for remaining amino groups by quantitative ninhydrin reaction; found 0.070 mmol. The concentration of amino groups remaining available for reaction at this stage on the resin should be one-half of the starting substitution.

Intersite Acylation To Form Peptide-Resin IV. Peptide-resin III (800 mg) was suspended in 15 mL of CH_2Cl_2 and activated by the addition of 0.070 mmol of HOBt and 0.070 mmol of DCC in 5 mL of CH_2Cl_2. After the solution was shaken for 12 h, the resin was filtered and washed and a 5-mg sample was withdrawn. This activation and coupling procedure was repeated four more times. The samples were dried and analyzed for amino groups by quantitative ninhydrin reaction. The amount of blue color slowly decreased with time, and the analysis was negative (<0.0003 mmol/g) after 60 h. In one preparation of the peptide-resin, even after repetitive DCC/HOBt and DCC/DMAP coupling steps, the ninhydrin analysis showed a constant level (0.032 mmol/g peptide-resin) of uncoupled amino group. In this instance, the reaction with 0.5 millimolar equiv ($^1/_2$ × 0.032 mmol/g peptide-resin = 0.016 mmol) of succinic anhydride was repeated, followed by DCC/HOBt coupling until the resin showed a negative ninhydrin test.

HF Cleavage of Peptide-Resin IV. The peptide-resin IV was dried on a vacuum pump for 3 h, and 50 mg was transferred to an HF reaction vessel. It was treated for 90 min at 0 °C with 4.5 mL of HF containing 0.25 mL of *p*-cresol and 0.25 mL of *p*-thiocresol. HF was distilled off under reduced pressure at 0 °C. The total time of exposure to HF was 2 h. The cleaved peptide was extracted into 10% HOAc (5 mL), and the resin was filtered on a sintered glass funnel. It was then washed with 10% HOAc (3 × 5 mL) and 20% HOAc (3 × 5 mL). All the aqueous

(27) The acylation was monitored by the ninhydrin reaction until only one-half of the starting peptide chains remained unreacted. It is essential at this stage not to allow the reaction to proceed further, or otherwise the product analysis would show an artificially high percentage of the monomeric tripeptides after HF cleavage of the peptide-resin.

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fractions were collected and extracted with diethyl ether (3 × 5 mL) to remove aromatic scavengers. The aqueous layer contained 0.0079 mmol of peptide by amino acid analysis (95%). It was lyophilized to yield 5.5 mg of free peptide.

Reduction of Liberated Peptide to Hexapeptide V. The free peptide (5.5 mg) obtained above was dissolved in 3 mL of 0.1 M NH_4OAc buffer, pH 8.5. N_2 was bubbled in slowly for 10 min and then 7.7 mg (0.05 mmol) of DTT in 1 mL of NH_4OAc buffer, pH 8.5, was added. The flask was stoppered and stirred at room temperature for 2 h. The pH was adjusted to 3 by the addition of 5% HOAc, and the reduced peptide, after lyophilization, was passed through a Sephadex G-15 column (elution solvent, H_2O). The column was monitored by absorbance at 225 nm, and the peptide under peak a, Figure 1 (30–54 mL), was pooled and lyophilized.

Oxidation to Cyclic Succinylbis[glutathione] (I). The disulfide bond was formed by dissolving the peptide in 60 mL of 0.1 M NH_4OAc, pH 8.2 (peptide concentration 0.13 mM), and stirring the solution in air. After 60 h it was Ellman negative. After lyophilization, the oxidized peptide was passed through a 1.8 × 40 cm Sephadex G-15 column and eluted with water (chromatogram B, Figure 1). A single component was obtained (volume to peak, 57 mL). Fractions of 48–65 mL were pooled and lyophilized; yield 5.1 mg (88% overall from NH_2CH_2 resin).

The product gave a single peak by ion-exchange chromatography on a 1 × 30 cm column of DEAE-Sephacel with a 0.05–0.75 M gradient of NH_4OAc, pH 6.8 (peak at 56 mL). It showed a single spot on TLC in three different solvent systems: R_f 0.42 in BuOH–pyridine–HOAc–H_2O (1:1:1:4); R_f 0.18 in BuOH–pyridine–HOAc–H_2O (5:5:1:4); R_f 0.1 in EtOAc–pyridine–HOAc–H_2O (5:5:1:1).

Fission-fragment mass spectrometry to 1600 mass units gave ions for $(M + Na)^+$ at 717.17 and $(M + 2Na - H)^+$ at 739.23 mass units, consistent with the expected 694.16 molecular weight. No dimer was detectable.

A sample was oxidized with performic acid to the sulfonic acid peptide and hydrolyzed in 6 N HCl. Amino acid analysis gave the following results: Gly, 1.03; Glu, 1.00; $CySO_3H$, 0.90.

Anal. Calcd for $C_{24}H_{34}N_6O_{14}S_2{\cdot}NH_3$: C, 40.51; H, 5.24; N, 13.77. Found: C, 40.88; H, 5.24; N, 13.77.

Synthesis of Peptide I by Solution Chemistry. Synthesis of VI. Glycine benzyl ester *p*-tosylate (1.59 g, 5 mmol) and triethylamine (0.5 g, 5 mmol) were mixed with 20 mL of DMF in a 125-mL erlenmyer flask. After the solution was stirred for 10 min at room temperature, N^{α}-Boc-*S*-benzyl-L-cysteine *N*-hydroxysuccinimide ester (2.12 g, 5 mmol) was added and stirring was continued for another 18 h at room temperature. The solvent was removed under reduced pressure, and the oily residue was taken up in 150 mL of ethyl acetate. The ethyl acetate solution was washed successively with 1 N HCl, H_2O, sodium carbonate buffer, pH 9.4, and H_2O. The organic layer was dried over magnesium sulfate and then flash evaporated to yield a semisolid, which crystallized as a white solid from ethyl acetate and petroleum ether: yield 1.85 g (82%); R_f 0.75; $CHCl_3$–MeOH (94:6); mp 77–78 °C.

Anal. Calcd for $C_{24}H_{30}N_2O_5S$: C, 62.86; H, 6.59; N, 6.11. Found: C, 62.87; H, 6.67; N, 6.24.

Synthesis of VII. The dipeptide VI (500 mg, 1.09 mmol) was stirred with 1.5 mL of F_3CCOOH at room temperature for 20 min. After evaporation of the acid by blowing N_2, 0.5 g of triethylamine in 5 mL of methylene chloride was added to the reaction flask and the solution was again stirred for 10 min. The solvent was flash evaporated and the residue dried under high vacuum for 3 h. The residue was dissolved in 5 mL of DMF and 474 mg (1.09 mmol) of Boc-L-glutamic acid α-benzyl-γ-*N*-hydroxysuccinimide ester was added. After the solution was stirred for 16 h at room temperature, DMF was evaporated and the residue was taken up in ethyl acetate. It was worked up as described for component VI. The solid so obtained from chloroform–petroleum ether precipitation showed two spots by TLC and was further purified through a 1 × 10 cm column of silica gel. The column was first eluted with chloroform (100 mL) and then with 1% acetic acid in chloroform. The desired peptide eluted in the latter solvent and showed a single spot on TLC. The solvent was flash evaporated, and the last traces of acetic acid were removed by evaporation with benzene three times. The white solid was dried in vacuo: yield 690 mg (93%); R_f 0.52 ($CHCl_3$–MeOH); mp 119–121 °C.

Anal. Calcd for $C_{36}H_{43}N_3SO_8$: C, 63.79; H, 6.39; N, 6.20. Found: C, 63.82; H, 6.37; N, 6.34.

Synthesis of VIII. A 120-mg sample of tripeptide VII (0.177 mmol) was treated with 1 mL of F_3CCOOH for 30 min at room temperature. After F_3CCOOH was evaporated with N_2, the residue was treated with 0.3 mL of triethylamine and worked up as described before. The dried residue was dissolved in 5 mL of DMF. The bis(*N*-hydroxysuccinimide) ester of succinic acid (27.7 mg, 0.088 mmol) prepared as described was added, and the solution was stirred overnight. DMF was removed under vacuum, and the oil was solidified by addition of ethyl acetate. Peptide VIII was further purified by elution from a column of silica gel with 4% MeOH in chloroform as the eluting solvent: yield 82.1 mg (63%); R_f 0.32 ($CHCl_3$:MeOH); mp 210–212 °C.

Anal. Calcd for $C_{66}H_{72}N_6O_{14}S_2$: C, 64.06; H, 5.86; N, 6.79. Found: C, 64.22; H, 5.84; N, 6.73.

Synthesis of the Reduced, Deprotected Hexapeptide V. The protected peptide VIII (110 mg) was treated with HF (10 mL) containing 0.5 mL of *p*-cresol and 0.5 mL of *p*-thiocresol[31] at 0 °C for 2 h. HF was evaporated at 0 °C, and the free peptide was extracted into 10% HOAc (3 × 5 mL) and 20% HOAc (3 × 5 mL). All the aqueous extracts were combined and washed with diethyl ether (3 × 20 mL). The aqueous layer was lyophilized to yield 80 mg of V.

Preparation of the Cyclic Hexapeptide I. Peptide V (70 mg) was dissolved in 5 mL of 0.1 M ammonium acetate buffer, pH 8.2, N_2 gas was bubbled in for 10 min, and then DTT (77 mg) in 1 mL of the buffer was added to reduce any disulfide that may have formed. The solution was stirred for 2 h and then brought to pH 3 with 5% acetic acid. It was lyophilized and then passed through a Sephadex G-15 column. Peptide was eluted with 5% aqueous acetic acid and was monitored by absorption at 254 nm. The desired hexapeptide fractions were collected and lyophilized to yield 60 mg of the reduced hexapeptide V.

The peptide obtained above was oxidized by air (60 h) in 150 mL of 0.1 M NH_4OAc buffer, pH 8.2. The solution was lyophilized and then passed through a column of Sephadex G-15. The desired oxidized peptide was eluted with 5% aqueous acetic acid and was monitored by absorption at 254 nm. The peak was centered at 57 mL. It was further purified by ion-exchange chromatography on a DEAE-Sephacel column using 0.05–0.75 M NH_4OAc buffer gradient, pH 6.8. The single peak (48–65 mL) was collected and lyophilized to yield 42 mg (45% overall from Gly-OBzl). It showed a single spot on TLC in three different solvent systems, with the same R_f value in each as found for compound I prepared by the solid-phase procedure. Within experimental error the amino acid analysis and elemental analysis were also the same as reported for the solid-phase preparation.

Discussion

The special design of this synthesis has allowed the succinyl-bis[glutathione] analogue I to be prepared conveniently and in high yield (88% from Boc-Gly-OMPA). When the coupling reactions were quantitatively monitored with a new ninhydrin method, each of them could be shown to have proceeded to within 0.3% of completion. This analytical method also allowed exactly half of the amino groups of the resin-bound tripeptide II to be acylated by succinic anhydride, with liberation of carboxyl groups exactly equal to the remaining amine. Activation as the *N*-hydroxybenzotriazole ester and subsequent aminolysis gave the cross-linked resin-bound cyclic hexapeptide by an intermolecular site–site interaction. This acylation was also followed to completion by the ninhydrin monitoring technique. Chromatographic analysis of the HF-cleaved product also showed that the site–site interaction was nearly quantitative (99.5%). The success of this approach was due to the absence of significant competing side reactions. The potential side reactions, all leading to the tripeptides, were (1) nonstoichiometric succinylation, leaving a small excess of either carboxyl or amino components, (2) rearrangement to inactive *N*-acylurea, (3) succinimide formation by intramolecular diacylation, and (4) incomplete activation or coupling to the succinyl carboxyl. All of these tripeptides could be easily separated from the desired hexapeptide by gel filtration on Sephadex G-15 (Figure 1, curve A), and the combined tripeptide fraction (peak c) was shown by hydrolysis and amino acid analysis to be only 0.5% of the total hexapeptide product (peaks a + b). No oligomers were expected or found. It should be noted that the chromatogram in Figure 1 was monitored at 225 nm, and in addition to the small amount of tripeptides several percent of UV-absorbing material was also present in peak c, which was derived from the aromatic scavengers used in the cleavage reaction.

The reduction step before the gel filtration was necessary because oxidized S–S dimers of tripeptides would have emerged with the succinyl hexapeptide and would not have been counted as tripeptide byproduct. The cleavage of peptide from the resin support was especially high (95%), but since it was not quanti-

(31) J. P. Tam, unpublished material.

tative, there may have been some bias in these results. However, because the hexapeptides were doubly bound to the resin support, we would have expected more, not less, to have remained uncleaved relative to the singly bound tripeptides, in which case the true extent of the site–site interaction would have been even higher.

The air oxidation of the reduced hexapeptide in dilute solution went readily and completely within 24 h. A single component was found on the Sephadex column (Figure 1, curve B). Although some oligomers were expected, none were found. The cyclic hexapeptide disulfide is more compact than the reduced peptide and was retained slightly longer. A standard mixture of GSH and GSSG showed that the latter, which has a lower molecular weight but is probably more extended than the oxidized succinyl peptide, comigrated with compound I. However, they were easily distinguished because compound I contained no SH and no NH_2, but after reduction contained free –SH and still migrated as a hexapeptide, whereas GSSG was reduced to the tripeptide. Compound I was also homogeneous by ion-exchange chromatography on DEAE-Sephacel and by TLC in several systems. Amino acid analysis, elemental analysis, NMR, and mass spectrometric analysis were all in agreement with the expected structure I. The product was also indistinguishable from a sample prepared from Gly-OBzl by solution chemistry.

The success of this solid-phase synthesis depended on each resin-bound peptide chain being able to reach another chain for reaction. Much experience has shown that no detectable amount of reaction occurs between beads. Therefore, the reacting chains were within the same resin bead and it was an intersite, or site–site, reaction. In the synthesis reported, the substitutions of active esters and of amino sites were each 0.09 mmol/g. In the reaction solvent dichloromethane, this resin swells to a volume of 5.8 mL/g.[21] Thus, with the assumption of random distribution of chains throughout the matrix,[32] the average distance between sites of attachment of reacting chains on the polystyrene backbone was ~48 Å at the start of the cross-linking reaction. The length of the fully extended amino-containing chains was ~24 Å, and in the ester chains, the distance to the activated carbonyl was ~29 Å. Therefore, at the beginning of the reaction some of the peptide chains would have been expected to collide and react due to their own random motion without invoking any flexibility or movement of the polymer backbone. However, as the reaction proceeded, the unreacted chains would, on average, have been farther and farther apart. At 90% reaction the distance would have been ~100 Å and at 99.5% it would have been ~275 Å, and reaction would not have been expected without significant polymer-chain motion.

Qualitatively this flexibility was, of course, expected to occur. Based on studies and on experience from our own laboratory, it is well recognized that polymer motion occurs in these solvent-swollen, lightly cross-linked polystyrene beads, and both intra- and intermolecular reactions can take place readily.[8-21] When more than one reaction is possible, the distribution of products and the proportion of intra- or intersite reactions are kinetically controlled.[14-16,20] Under certain circumstances (high cross-linking, low substitution, nonswelling solvent, charged substituents) the effective separation of resin-bound functional sites can be observed and utilized to advantage.

Site–site interaction has been quantitated in several instances. In their studies on the oxidation of resin-bound Boc-Cys-Gly-resin, Lunkenheimer and Zahn[11] showed that, at a loading of 0.5 mmol/g, up to 75% of the sulfhydryl groups could be closed to the disulfide by Fe^{+3}-catalyzed air oxidation in Me_2SO/CH_2Cl_2. Crowley and Rapoport[14] showed that Dieckmann reaction of ω-cyanopelargonyl thio resin ester (0.4 mmol/g) gave 10–19% of dimeric diketo dinitrile and 5% or less of the intramolecular cyclization product 2-cyanocyclononanone. These same authors also showed that the formation of anhydrides from (carboxymethyl)styrene–2% divinylbenzene resin could be observed in solvent-swollen beads. At 0.05 mmol/g, 53% of the resin-bound carboxyl groups could be converted to intersite anhydrides, and at high loading (~1 mmol/g), up to 80% of intersite product was obtained. Kraus and Patchornik[15] demonstrated 35% of base-catalyzed site–site condensation in a 2% cross-linked resin containing 0.6 mmol/g of nonenolizable *p*-chlorobenzoate ester and 0.2 mmol/g of enolizable octanoate ester. By heavily loading (1.7 mmol/g) with a nonenolizable ester, the limiting enolizable ester could be reduced from 0.07 to 0.005 mmol/g. However, this does not demonstrate polymer backbone flexibility because the active anions were always close to other ester groups that remained in high concentration. Bifunctional reagents HOC_6H_4OH,[6] $I(CH_2)_4I$,[18] and $HS(CH_2)_nSH$[19] have all been used to demonstrate site–site interaction, although the experiments were complicated by the presence of competing side reactions. Thus, Farrall and Fréchet[19] were able to obtain a 94% site–site yield between $ClCH_2$-resin and $HS(CH_2)_4SH$, with only 0.01 mmol/g of unreacted monosubstituted resin remaining at the end of the reaction. Wulff[10] found 69% oxidation of resin SH to disulfide in a low-cross-linked, random copolymer at 0.8 mmol/g.

We were interested in determining quantitatively how completely an intersite reaction could proceed; i.e., how low could the substitution of functional groups drop and still allow demonstrable reaction. The present data show that reaction occurred down to less than 0.0005 mmol/g (~10^{-4} M) of each species, equivalent to only 1 functionallized styrene in in 20 000. This is 40 times fewer unreacted sites than found in the carboxymethyl-resin anhydride experiment[14] and 20-fold lower than in the thiol displacement of chloromethyl groups.[19] We think this means that very extensive polymer-chain flexibility occurs in low-cross-linked, solvent-swollen polystyrene beads, with segment movement of at least 200 Å, and that nearly all functional sites have sufficient mobility to encounter another reactive site within the same polymer bead.

Abbreviations used: Boc, *tert*-butyloxycarbonyl; Pam, (phenylacetamido)methyl; DCC, *N,N'*-dicyclohexylcarbodiimide; HOBt, 1-hydroxybenzotriazole hydrate; TFA, trifluoroacetic acid; DIEA, diisopropylethylamine; DMF, *N,N*-dimethylformamide; 4-MeBzl, 4-methylbenzyl; DMAP, 4-(dimethylamino)pyridine; GSSG, oxidized glutathione; DTT, dithiothreitol.

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(32) R. B. Merrifield, and V. Littau, in "Peptides 1968", E. Bricas, Ed., North Holland, Amsterdam, 1968, p 179.