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Peptide synthesis using unprotected peptides through orthogonal coupling methods

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ABSTRACT **We describe an approach to the synthesis of peptides from segments bearing no protecting groups through an orthogonal coupling method to capture the acyl segment as a thioester that then undergoes an intramolecular acyl transfer to the amine component with formation of a peptide bond. Two orthogonal coupling methods to give the covalent ester intermediate were achieved by either a thiol–thioester exchange mediated by a trialkylphosphine and an alkylthiol or a thioesterification by C^{α}-thiocarboxylic acid reacting with a β-bromo amino acid. With this approach, unprotected segments ranging from 4 to 37 residues were coupled in aqueous solution to give free peptides up to 54 residues long with high efficiency.**

Recent advances in the design of artificial proteins with unusual architectures (1) and the ready availability of large peptides and protein domains from both solid-phase synthesis (2, 3) and recombinant DNA have led to the need for the development of convergent strategies in peptide synthesis with these large unprotected peptide segments as building blocks. A requirement of such a strategy is the exceptionally high regiospecificity in the amide bond formation between the α-amine and C^{α}-acyl moiety of the two segments. Recently, we have proposed (4–7) an orthogonal coupling method to achieve this goal.

An orthogonal coupling method to form an amide bond is conceptually different from an orthogonal protecting group strategy (8). It is site-specific and allows only a single specific coupling reaction between the C^{α} moiety of one peptide segment and the N^{α}-amine of another peptide segment, in the presence of other reactive amino moieties. In such a method, a capture step (9) brings the respective N and C termini into close proximity and the amide bond formation is effected by an intramolecular acyl transfer at high effective concentration. So far, all orthogonal couplings have exploited the unique reactivity of the 1,2-aminothiol moiety of an N-terminal cysteine on the amine segment to achieve selectivity to form thiazolidine, thioester, and disulfide with acyl segments bearing glycolaldehyde ester (4, 5), thioester (6, 10), and mixed disulfide (6, 7). In orthogonal couplings involving C^{α}-thioester and N^{α}-cysteine, the capture step occurs when the covalent thioester is formed between these two segments leading to a spontaneous S- to N-acyl transfer to form a cysteinyl peptide bond (Fig. 1). In this paper, we describe two orthogonal coupling methods involving a thioester intermediate to obtain peptides with a cysteinyl bond and further clarify the mechanism by which this occurs. The first method involves the thiol side chain of an N-terminal cysteine to initiate a nucleophilic attack on a thioester segment to give a covalent thioester intermediate under special reductive conditions with a trialkylphosphine and excess alkylthiol. The second method involves a nucleophilic attack in the reverse direction, via a C^{α}-thiocarboxylic acid of an acyl segment that S-alkylates an N-terminal β-bromoalanine (BrAla) and forms a similar covalent thioester that will rearrange rapidly via an intramolecular S-to-N acyl transfer to give the peptide bond (Fig. 1).

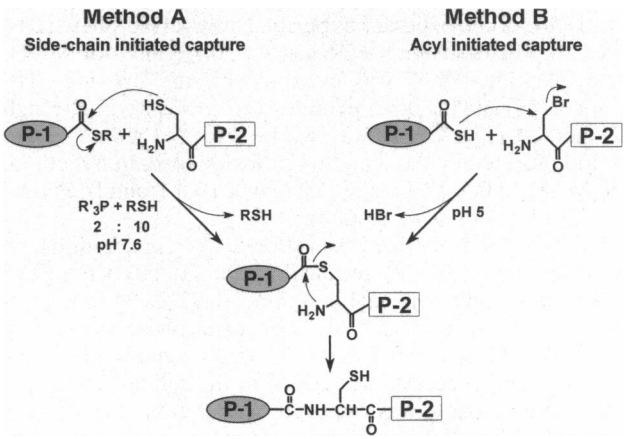

FIG. 1. Schematic pathway for the proposed orthogonal coupling using fully unprotected peptide segments. The capture is effected by a thiol–thioester exchange in method A and thioesterification in method B. Both will give a covalent thioester leading to a spontaneous intramolecular acyl transfer to give a cysteinyl peptide bond. P-1 (shaded oval), peptide-1; P-2, peptide-2 (open square); R, $CH_2CH_2CO_2H$.

EXPERIMENTAL PROCEDURES

General Method. Solid-phase peptide synthesis was performed manually or on an Applied Biosystems model 430A synthesizer. Analytical HPLC was run on a Shimadzu system with a Vydac (Hesperia, CA) column (0.46 cm × 25 cm, C_{18} reversed phase, 5 μm) using linear gradients of buffer B [60% (vol/vol) acetonitrile in H_2O/0.04% trifluoroacetic acid (TFA)] in buffer A (5% acetonitrile in H_2O/0.045% TFA), with UV detection at 225 nm and at a flow rate of 1.0 ml/min or 1.5 ml/min. Mass spectra were obtained with the matrix-assisted laser desorption mass spectrometry. The calculated mass units were given as average isotopic values. *tert*-Butoxycarbonyl (Boc)- and fluorenylmethoxycarbonyl (Fmoc)-amino acid derivatives were purchased from Bachem.

Solid-Phase Peptide Synthesis. Peptides, CA-4F and BV-7 (see Table 3), were synthesized by the Fmoc/*t*-Bu strategy on Wang resin (11) by using the benzotriazol-1-yl-oxy-tris-(dimethylamino)-phosphonium hexafluorophosphate (BOP) coupling protocol and cleavage by 95% TFA/4% H_2O/1% triisopropylsilane. Thioester, thiocarboxylic peptides, and CA-17 were prepared by the Boc/benzyl strategy using either the BOP coupling protocol (12) or the dicyclohexyl carbodiimide/hydroxybenzotriazole method. CA-17 was synthesized from Boc-Ala-OCH_2-phenylacetamidomethyl resin (13). The

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Abbreviations: BrAla, bromoalanine; Boc, *tert*-butoxycarbonyl; DMF, dimethylformamide; Fmoc, fluorenylmethoxycarbonyl; RP-HPLC, reversed-phase HPLC; TFA, trifluoroacetic acid; R, propionic acid; Leu-SH, thioleucine; Acm, acetamidomethyl.

synthesis of peptide SG-5 was started with coupling of Boc-Gly-S$(CH_2)_2$COOH onto *p*-methyl benzhydrylamine resin while GL-6 and PN-37 were started with coupling of Boc-Leu-S$(CH_2)_2$COOH or Boc-Asn-S$(CH_2)_2$COOH onto H-Gly-OCH_2-phenylacetamidomethyl resin, respectively (14). The dinitrophenyl protecting group on His residues was removed before HF cleavage by 10% (vol/vol) thiophenol/2.5% (vol/vol) *N,N*-diisopropylethylamine in dimethylformamide (DMF) (for one 2-h period and two 8-h periods). Ser-Ala-Lys-Leu-SH, where Leu-SH is thioleucine, was synthesized on the handle of 4-[(Boc-Leu-S)benzyl]phenoxyacetic acid (15) by using Boc/benzyl chemistry. After HF cleavage with 9:1 (vol/vol) HF/anisole, the crude peptide was purified by reversed-phase (RP) HPLC. Laser desorption MS: 434.3 ± 0.4 (calcd for M+H, 434.6) Peptide PN-37 was cleaved with the low–high HF protocol (16) and the other peptides were cleaved with the high HF method (17). CA-17 was synthesized and stored as the disulfide formed by the dimethyl sulfoxide oxidation method. MS $[M+H]^+$: CA-17, *m/z* 1801.0 (calc), 1801 (found); PN-37, *m/z* 4281.8 (calc), 4282 (found).

Boc-BrAla was obtained from Boc-Ser (4.1 g, 20 mmol) in dichloromethane (50 ml), tetrahydrofuran (10 ml), CBr_4 (9.95 g, 30 mmol), and triphenylphosphine (8.92 g, 34 mmol) as described by Hayashi *et al.* (18). The organic phase was washed with H_2O, saturated NaCl, and H_2O. After removal of dichloromethane, ethyl acetate was added to the residue and triphenylphosphine oxide was removed by filtration. The residue was purified by silica gel column chromatography using hexane/ethyl acetate/acetic acid, 60:40:1 (vol/vol), as eluent. Yield: 1.68 g (31.3%). BrAla-Pro-Gly-Gly-Asn-Cys(Acm)-Val-OH, where Acm is acetamidomethyl, was prepared by Fmoc chemistry on Wang resin using the dicyclohexyl carbodiimide/butanol method. The peptide was cleaved from the resin with 95% TFA/anisole for 30 min. After removal of TFA, the peptide was precipitated with dry ethyl ether and washed with dry ether. The crude peptide was purified with RP-HPLC. Laser desorption MS: 767.6 ± 0.7 (calcd for M+H, 767.7).

General Reaction of Thioesterification Through Thiocarboxylic Acid and BrAla. Leu-SH and BrAla were obtained after removal of Boc protection with TFA. Thioesterification in 50% DMF/BrAla-OH (25 μl, 0.08 M) in DMF (100 μl) was added to aqueous solutions of Leu-SH (25 μl, 0.08 M) and buffered at pH 4.7, 5.2, 5.7 (sodium acetate), and 6.2 (sodium phosphate). The final concentrations of Leu-SH and BrAla-OH were 0.01 M and analyzed by RP-HPLC and laser desorption MS. The desired dipeptide Leu-Cys (retention time, 7.1 min) and isomer **11** (see Fig. 4) (retention time, 9.2 min) were obtained. Both products had the same molecular weight as Leu-Cys. The percentage of **11** increased as the pH of reaction solution increased (pH 4.7, 3%; pH 5.2, 18%; pH 5.7, 28%; pH 6.2, 40%). The thioesterification was complete in <15 h. Similarly, ligation in water at pH 5.6, 6.0, and 6.7 was studied with Leu-SH (50 μl, 0.04 M) and BrAla (50 μl, 0.04 M). Compounds **8** and **11** in similar ratio were formed more slowly than in 50% DMF.

Table 1. Effect of pH on product distribution in thiol–thioester exchange in 18 h

	Acylated products, mol %			
pH	α (1)	α,S (2)	α,SSR[1] (3)	Boc-Gly-OH
5.6	5	2.5	12	78
6.6	43	3	20	34
7.2	44	3	16	38
7.6	56	4	25	15

Acylated products on α and thiol (S) (compounds **1** and **2**; see Fig. 2 for structure), α-acylated and thiol as mixed disulfide (compound **3**), and hydrolysis product (Boc-Gly-OH).

Table 2. Effect of alkyl thiol and R_3P on thiol–thioester exchange of Boc-Gly-SR and Cys-Phe-Lys-Ala(CA-4F) in 8 h at pH 7.2

			Yield, mol %		
			Acylated products		Mixed disulfide
Entry	Reagent	Equiv.	α(1)	α,S(2)	α,SSR[1](3)
1	None		44	4	17
2	R_3P	2	87	13	—
3		4	75	25	—
4	R_1SH	1	35	14	51
5		5	73	1	26
6		10	82	—	18
7	R_3P + R_1SH	2:2	80	6	14
8		5:5	89	3	8
9		2:5	95	3	2
10		2:10	98	<1	1

Hydrolysis products of Boc-Gly <2%, except in entry 1 are shown. R, CH_2CH_2COOH; R_1, CH_2CH_2COOH.

Model Reaction of Thioester Peptides with Cys Peptides. Boc-Gly-SR (where R is propionic acid) and CA-4I (CA-4 or other cysteinyl-containing tetrapeptides) in equal molar ratio (1.0 μM) with various additives (Tables 1 and 2) in a buffered routine of pH 5.6, 6.6, 7.2, and 7.6 were allowed to react at predetermined intervals at ambient temperature. Yields and product distribution were analyzed by RP-HPLC using a linear gradient of 0–85% buffer B in 30 min. Retention times (in min) for products (see Fig. 2) were as follows: **1** 22.7; **2** 27.4; **3** 23.5 and 26; **4** 14.2. α-, ε-, and α- and ε-acylated peptides were confirmed by independent synthesis. Matrix-assisted laser desorption mass spectrometry: **1** 622.74 (calcd), 623.4 (found).

Thiol–Thioester Exchange of PN-37 with CA-17. PN-37 (0.43 mg, 1×10^{-4} mmol) and CA-17 (0.45 mg, 2.5×10^{-4} mmol) were first dissolved in 50 μl of buffer B in a small (0.5 ml) plastic vial. Tris(2-carboxyethyl)phosphine (0.17 mg, 6×10^{-4} mmol) and *N*-hydroxysuccinimide as a weak acid buffer (≈0.1 mg, 8.7×10^{-4} mmol) were then added. The pH was adjusted to ≈6.5 by addition of solid sodium acetate. RP-HPLC monitoring showed that ≈60% of ligation product was formed (peak 2 in Fig. 3); the peak corresponding to the starting material (peak 1) was found to contain around one-third of the hydrolysis product of the thioester as confirmed by MS. The identity of the broad peak at 23.5 min was not clear, since no detectable species were found by MS analysis. HPLC gradient was 40–75% buffer B for 35 min. Peptide CA-17 was eluted immediately with the injection peak. MS $[M+H]^+$: ligation product (PA-54), *m/z* 5920.8 (calc), 5919.6 (found); hydrolysis product of PN-37, *m/z* 4136.8 (calc), 4136 (found).

RESULTS

Preparation of Cysteinyl and Thioester Segments. Unprotected amine segments containing an N-terminal cysteinyl residue were synthesized by the conventional solid-phase method (3) using either Boc or Fmoc chemistry. Thioester and thiocarboxylic acid segments were also prepared by the stepwise solid-phase synthesis using thioester resin developed by Hojo and Aimoto (14) and Yamashiro and Li (15). Because of the lability to amine nucleophiles in the Fmoc chemistry, thioesters or thiocarboxylic segments were prepared by Boc chemistry, cleaved from the resin by a modified low–high HF (16), and purified by RP-HPLC. All peptide segments were vigorously characterized by several methods, including MS analyses, and gave the expected results.

Model Reactions to Study Selectivity and Product Distribution of the Thiol–Thioester Exchange. The rates, yields, and product distributions of the thiol–thioester exchange were studied at four discrete pH values (pH 5.6, 6.6, 7.2, and 7.6)

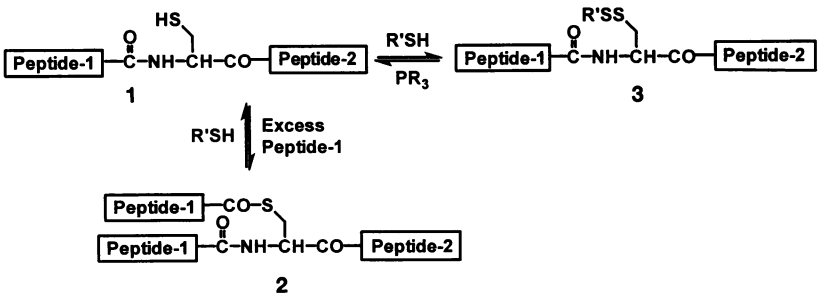

FIG. 2. Major products and their interconversion in the model reaction of CA-4F and Boc-Gly-SR. Compound **3** represents products with mixed disulfide where R^1 is CA-4F, **1**, or thiopropionic acid.

with a model peptide, CFKA, and Boc-Gly-SR. Because of the presence of thiols in both starting material and products, there are 16 possible products even with this simple model reaction, 11 of which would contain disulfides with one of the thiol-containing compounds: CFKA, Boc-Gly-CFKA, and thiopropionic acid derived from Boc-Gly-SR (Fig. 2). Rates of the thiol–thioester exchange increased 1.5- to 2-fold with each unit increment of pH as the anionic thiolate concentration increased (data not shown). The reaction was largely complete at pH 7.6 in 12 h. However, three groups of byproducts were also obtained: α,S-diacylated **2**, a mixture of α-acylated product with disulfide **3**, and the hydrolysis product, Boc-Gly (Table 1). At pH 5.6, the reaction was slowest and hydrolysis of the thioester greatest, 78%, while at pH 7.6, hydrolysis was lowest to yield 56% of the desired product **1**, indicating that the thiolate could significantly compete with hydrolysis at high pH values.

These results led us to consider the use of a basic pH to inhibit hydrolysis of the thioester and a strong reducing environment of an alkyl thiol and trialkylphosphine to prevent α,S-diacylated product **2** and to reduce disulfide formation. R_3P was found to reduce disulfides and eliminate the disulfide byproducts **3** (Table 2, entries 2 and 3), while the excess alkylthiol converted the α,S-diacyl byproduct **2** to the starting material **1** (Table 2, entries 4–6). Indeed, a combination of R_3P and a large excess of an alkyl thiol improved the yield to >95%, with nearly no detectable hydrolysis of Boc-Gly-SR and few other byproducts (Table 2, entries 9 and 10), and appears to be an optimal condition for the thiol–thioester exchange. The use of reduced acylthiols, such as reduced Ellman's reagent, was found to be ineffective because they existed mostly in their thione forms (data not shown). We found that a water-soluble phosphine, tris(2-carboxyethyl)phosphine, was convenient to use, but others such as triethylphosphine were also effective. There is evidence that R_3P activates the thioester as a phosphonium salt to accelerate the thiol–thioester exchange since the reaction was largely complete in 4–8 h at pH 7.6 and because R_3P promoted α,S-acylated byproducts (Table 2, entries 2–4 and 7). The combination of R_3P and RSH was also studied at pH 6.6 and 7.6 with both peptides CA-4 and CA-4F (Table 3) and similar results and product distributions were obtained (data not shown).

The selectivity of aminolysis between α- and ε-amine was also studied with model tetrapeptides CKFA, CAKA, and CFKA, containing a lysinyl amine near the N terminus to compete for the acylation reaction. At pH 7.6 or lower, ε-acylation was <2%, indicating the reaction is α-amine-specific. Although the side reaction due to hydrolysis in Boc-Gly-SR was no longer a significant problem with our proposed conditions, we found that it was still relatively significant with trifunctional amino acids whose side chain participated in assisted hydrolysis as in the case of PN-37 (Table 3), which contained a terminal Asn-SR. The covalent thioester intermediates were not detectable by RP-HPLC because of their rapid S-to-N intramolecular acyl transfer. However, the proposed reaction scheme is consistent with the thiol side chain-initiated nucleophilic attack of the thioester rather than direct acylation by the α-amine. Tetrapeptides of S-protected CA-4 [Cys(Acm)-AKA], SAKA, GAKA, and LAKA did not give any N^{α}- or N^{ε}-acylation in 18 h at pH 6.6–7.6.

Application in Peptide Synthesis. Using these optimized conditions, we prepared four peptides ranging from 9 to 54 amino acids with 60–88% yield (Table 3). The synthesis of a 39-residue peptide, AA-39 (Table 3), serves to illustrate our proposed conditions for the orthogonal coupling. The amine segment CA-22 and tris(2-carboxyethyl)phosphine (1:2 molar ratio) were mixed with the acyl segment thioester AS-17 and mercaptopropionic acid (1:10 molar ratio) in a phosphate-buffered solution at pH 7.6 and allowed to react 36–48 h to give the desired peptide AA-39 in 60% yield. The synthesis of another 54-residue peptide was equally efficient and noteworthy because an internal cysteine was unprotected and the coupling gave cleanly separable peaks for easy purification (Fig. 3).

Table 3. Peptides synthesized by orthogonal coupling through thioester capture

Peptide	Acyl segment	Peptide	Amine segment	Method	Product	Yield, %
SG-5	SRDFG*	CA-4	CAKA	A	SA-9	88
GL-6	GERGAL*	CA-4F	CFKA	A	GA-10	87
AS-17	AVSEINFMHNLGKHLSS*	CA-22	CDHARHGFLPRHRDTGI-LDSC(Acm)A	A	AA-39	60
PN-37	PQITLWQRPLVTIRIGGQL-KEALLDTGADDTVLEEMN*	CA-17	CHSGYVGARCEHADLLA	A	PA-54	60†
SL-4	SAKL‡	BV-7	BPGGNAC(Acm)V§	B	SV-11	85

Methods A and B are as in Fig. 1. The yield is based on amine segment, with acyl segment in 1.2- to 1.5-fold excess, and calculated from area ratio corresponding to peaks of amine segment and product.

*As thioester.

†In PN-37, yield was based on acyl segment with amine segment in 2.5-fold excess.

‡As thiocarboxylic acid.

§B (in sequence), BrAla.

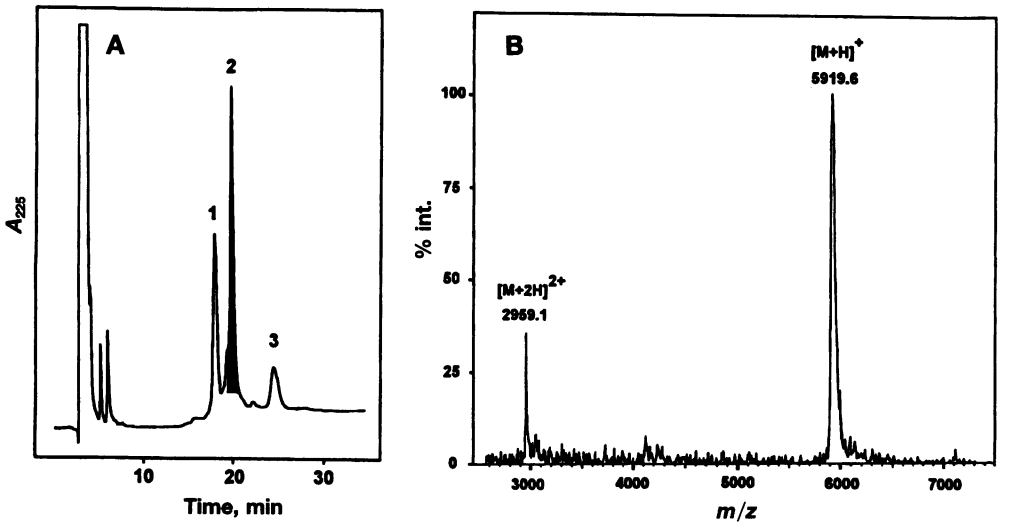

FIG. 3. (*A*) RP-HPLC of coupling PN-37 (peak 1) and CA-17 (eluted near solvent front) by thiol–thioester exchange to give PA-54 (peak 2) after 12 h. Peak 3 is an artefact. (*B*) Matrix-assisted laser desorption mass spectrometry of PA-54. int., Intensity.

Thioesterification Through Thiocarboxylic Acid and BrAla. Another orthogonal method to couple acyl and amine segments via thioesterification (19) was studied (Fig. 1, method B). We envisioned that a reverse directional nucleophilic capture of BrAla by a C-terminal thiocarboxylic acid would also yield a covalent thioester similar to the one shown in Fig. 1, method A. BrAla was obtained in good yield by triphenylphosphine-mediated bromination of Boc-Ser in solution (18) or in solid phase. Boc-BrAla was incorporated in a stepwise solid-phase synthesis scheme without difficulty because it was the N-terminal amino acid residue as shown in the synthesis of BV-7 (Table 3).

For model reactions, we studied the thioesterification of Leu-SH with BrAla at pH 5.2–6.2 and two products with identical molecular weight were obtained, the desired α-dipeptide **8** and its β-isomer **11** derived from the thiocarboxylic attack at the α-position of the aziridine ring (Fig. 4). The formation of an aziridine ring of an N-terminal BrAla peptide was due to the ease of 1,3-elimination of HBr, the highly labile proton on the α-amine. Subsequent ring opening at the α or β position by the thiocarboxylate to give α or β peptides was expected to be facile due to the ring strain. Aziridine formation (20) was favored at pH >6 and under such a condition BrAla was converted to aziridine in 1–2 h. Thiolysis of aziridine or BrAla at pH >6 gave compounds **8** and **11** in a ratio of 6:4 favoring ring opening at the less-hindered β position (21, 22). If this occurs, the α-thio-β-alanine-containing byproduct **11** must be removed by suitable purification procedures. This byproduct **11** was minimized to <3% when the thioesterification was conducted at pH <5 when the aziridine formation was slow and the dominant thioesterification occurred by direct displacement of BrAla.

BrAla is prone to β-elimination to give dehydroalanine and the thioesterification could occur by the Michael addition of the thiocarboxylic acid. This mechanism was ruled out because there was no racemization after comparing with authentic D-Leu-Cys and Leu-D-Cys samples in RP-HPLC. Furthermore, β-elimination of N-terminal BrAla only occurred at pH >11 with rapid decomposition to pyruvic acid. The feasibility of the thioesterification in our orthogonal coupling method and intramolecular aminolysis was validated in the synthesis of sperm-activating peptide in 85% yield (Table 3).

DISCUSSION

There are three elements in our approach: (*i*) the use of fully unprotected peptides, (*ii*) orthogonal coupling methods, and (*iii*) intramolecular acyl transfer. The use of unprotected peptide segments or protein domains as building blocks presents significant advantages and various challenges. Advantages include aqueous solubility, accessibility to protein purification methods, and readily available sources of building blocks derived from solid-phase peptide synthesis and recombinant DNA methodologies. More importantly, large unprotected peptides are likely folded into ordered structures, which offer solvent-exposed N and C termini and the possibility of conformational assistance (21, 22) by placing two segments in close proximity to overcome entropy in effecting the amide formation. Finally, there is no strong acid or other harsh treatment to denature the products after the coupling reaction

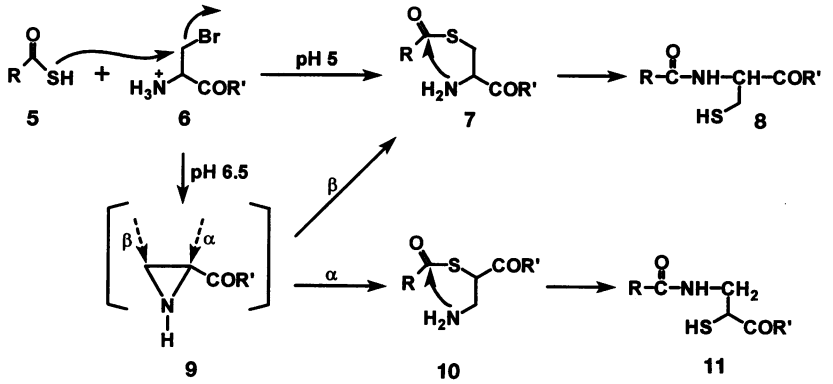

FIG. 4. Mechanism and products of thioesterification by thiocarboxylic acid and BrAla and the aziridine intermediate **9**.

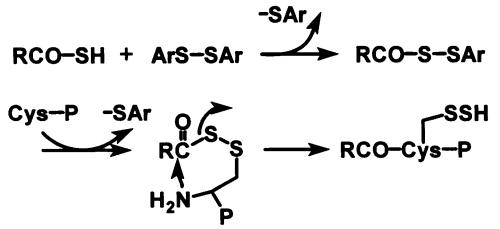

FIG. 5. Mechanism of orthogonal coupling by thiol–disulfide exchange via thiocarboxylic activated by Ellman's reagent (ArS-SAr). R and P, unprotected peptides; Ar, aryl substituent.

since there are no protecting groups to be removed. These advantages are usually not found in the conventional convergent approaches (23–28) that use partially or fully protected peptide segments.

The use of unprotected peptides also necessitates the development of methods to achieve high regioselectivity in peptide bond formation. A coupling reaction with such regioselectivity would require orthogonality by a capture step to form a covalent thioester between the acyl and amine segments subsequently leading to a proximity-driven S-to-N intramolecular acyl transfer through a five-member intermediate to give a peptide bond (Fig. 1). Our results show that this can be achieved efficiently by two methods. (*i*) It can be formed by a nucleophilic attack of the side-chain thiol of a cysteinyl N-terminal segment on a C-terminal thioester. This reaction becomes highly efficient in a strongly reducing and nucleophilic environment containing a combination of trialkylphosphine and alkyl thiol. R_3P eliminates byproducts due to disulfide formation and accelerates the thiol–thioester exchange while the alkyl thiol converts the S-acyl byproducts to the desired product and the starting material. (*ii*) The thioester can also be formed in the reverse direction through the nucleophilic attack of thiocarboxylic acid of the acyl segment on BrAla of the amine segment. BrAla or its active aziridine form provides an alternative approach for preparing amine segments without the use of N-terminal cysteine. It may be useful when incorporating segments containing disulfides or thioester linkages.

The thiol–thioester exchange also illustrates the concept of the orthogonal coupling method. Thioesters are relatively stable to amines but reactive to thiols under aqueous conditions at pH 5–7.5. Under our proposed conditions containing R_3P and excess alkylthiol, there is no productive thiol capture between thioester and those thiols occurring as cysteinyl side chains in the internal sequence of either the amine or acyl segments, as shown in Table 3, but this becomes productive with a 1,2-aminothiol structure, which occurs only in the N terminus of the amine segment. Thus, even though the acylated thioester could react with many thiols, only N-terminal cysteine will yield a single thermodynamically stable product. A similar concept of orthogonality in coupling reactions has been exploited by our laboratory in capture steps mediated by thiazolidine and oxazolidine ring (4, 5) as well as disulfide formation (6, 7).

Recently, a scheme based on thioester exchange was reported (10) that was a C^{α}-thiocarboxylic acid, Ellman's reagent, and cysteinyl thiol at basic pH. We (6, 7) have shown that this scheme does not proceed through a thiol–thioester exchange but, rather, through two sequential disulfide exchanges, an acyl mixed disulfide with the Ellman's reagent and a subsequent disulfide exchange with cysteinyl thiol. The latter covalent acyl disulfide eventually leads to an intramolecular acyl transfer involving a six-member intermediate containing a disulfide, rather than a five-member intermediate of a thioester (Fig. 5), to yield a peptide containing a hydrodisulfide on the cysteine.

Intramolecular acyl transfer as a mechanism to obtain high effective molarity (estimated to be as high as 10 M) and to overcome entropy in coupling two peptide segments of high molecular weights has been advocated by Kemp and Carey (9, 29). Interconversions of intramolecular acyl transfers are well known in peptide synthesis as side reactions (17, 30) and have been found as significant cellular processes of posttranslational protein splicing (31). Thus, the combination of orthogonal coupling through the N-terminal side chain and a subsequent intramolecular acyl transfer could be considered a bioorganic approach to mimic the natural process in peptide synthesis and holds potential promise for synthesis of large peptides and semisynthesis of proteins.

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