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High-performance liquid chromatographic investigations on the cleavage kinetics of side-chain-protected arginine derivatives with a sophisticated post-column reaction detector

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ABSTRACT

In the field of peptide synthesis, only a few exact data on the behaviour of side-chain-protecting groups and their specific cleavage methods are available. A method is described for the separation and highly specific detection of guanidino-protected and -free arginine residues. The simultaneous determination of the cleavage kinetics of α- and side-chain-protecting groups of arginine becomes feasible. A high-performance liquid chromatographic system with a highly specific post-column reaction detector for the (released) guanidino group in arginine derivatives was developed. The cleavage kinetics of the most important protecting groups for the guanidino residue in arginine derivatives were established.

INTRODUCTION

Various methods in protein chemistry, *e.g.*, classical and solid-phase synthesis and the modification of natural peptides and proteins, require side-chain-protected amino acid derivatives. The aim is to use appropriate protecting groups that resist all the required chemical and biochemical treatments but on the other side can be cleaved under special conditions. One of the most difficult side-chain groups to protect is the guanidino group in arginine, which shows additionally a great tendency to form lactams. To optimize the strategy for the synthesis or chemical modification of peptides or proteins containing guanidino-protected arginine residues it is important to consider the reactivity and the cleavage kinetics of the applied side-chain-protecting groups in relation to the optimum mode of synthesis.

To control the cleavage of guanidino-protecting groups, several methods have been described[1–4]. However, almost no data about the specificity of the reaction, including the side-reactions, are available.

An increase in the overall yield of a synthesis and the minimization of the side-reactions require an exact knowledge of the reaction kinetics of all the important

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steps. In the case of arginine derivatives, almost no data are available. Samples of synthetic peptides which have been submitted to the mostly used cleavage conditions show, on high-performance liquid chromatography (HPLC) numerous peaks resulting from the reaction itself, and from the reagents and solvents. Hence a precise determination of the released guanidino group is virtually impossible. This prompted us to develop a post-column reaction detector with high specificity with respect to the guanidino group in arginine residues.

Phenanthrenequinone reacts with the guanidino group in arginine specifically to form a fluorescent dye[5,6]. Fig. 1 shows the proposed mechanism of the reaction of phenanthrenequione with arginine in alkaline solution and the production of a Schiff base, 2-amino-1*H*-phenanthro[9,10-*d*]imidazole (API). The eluted components are first detected with a standard UV detector. There after an alcoholic solution of phenanthrenequione is added, reacted in alkaline conditions and finally acidified to form the fluorescent compound, which is detected with a fluorescence monitor. High selectivity and sensitivity are found[7,8]. Hence it is possible to determine the cleavage kinetics of allmost all guanidino-protecting groups used in peptide chemistry. The developed separation technique allowed us to investigate and to evaluate the suitability of the most frequently used guanidino protecting groups, *viz.*, 4-methoxy-2,3,6-trimethylbenzenesulphonyl- (Mtr)[9], 2,2,5,7,8-pentamethylchromane-6-sulphonyl- (Pmc)[10], *p*-methoxybenzenesulphonyl- (Mbs)[11], mesitylene-2-sulphonyl- (Mts)[12] and 2,4,6-triisopropylbenzenesulphonyl- (Tip)[13].

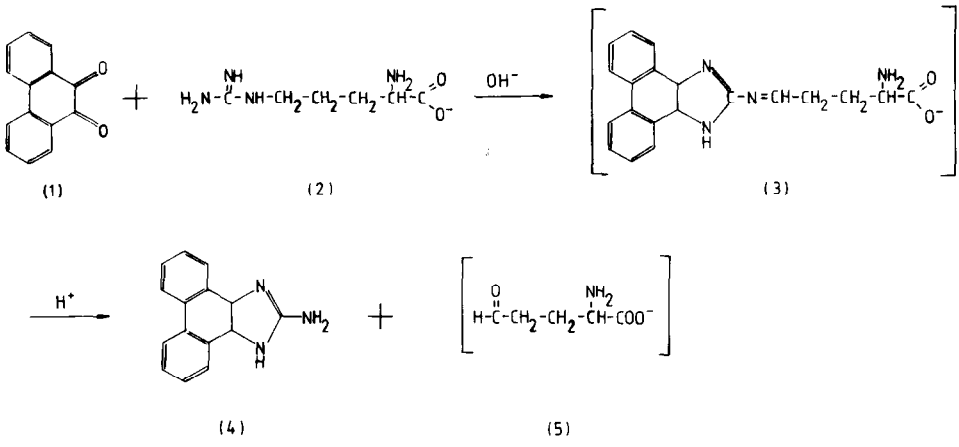

Fig. 1. Formation of the fluorescent dye from 9,10-phenanthrenequinone with the guanidino function of arginine. (1) 9,10-Phenathrenequinone; (2) arginine; (3) 2-(ω-imidoglutamic acid)-1*H*-phenanthro[9,10-*d*]imidazole; (4) 2-amino-1*H*-phenanthro[9,10-*d*]imidazole (API); (5) glutamic-γ-semialdehyde.

EXPERIMENTAL

Apparatus

The investigations were performed with a modified Biotronik BT 3020 liquid chromatograph. The separation was carried out on a 250 × 4 mm I.D. steel column of Nucleosil 100-C_{18}(5 μm). Direct detection was effected with a standard variable-wavelength UV monitor. The effluent from the UV detector was continuously mixed

with 1 mol l^{-1} sodium hydroxide solution and thereafter with a solution of 2.5 mg l^{-1} pheneanthrenequinone in ethanol. The reaction of eluted guanidino residues to give a Schiff base (see Fig. 1) was performed in a 16 m × 0.25 mm I.D. PTFE tube at elevated temperature (95°C). The fluorescent dye was finally formed in a second 4 m × 0.25 mm I.D. PTFE tube on adding 5% sulphuric acid at ambient temperature. Detection was carried out with an F1000 fluorescence monitor (313/395 nm) (Merck–Hitachi, Darmstadt, F.R.G.).

Chemicals and reagents

Amino acid derivatives of the highest available purity were obtained from Novabiochem (Läufelfingen, Switzerland). (*Z*)-Arg(Tip)-OH was synthesized in our laboratory. All other solvents and reagents were of analytical-reagent of HPLC grade from Merck.

Evaluation of the eluent and reagent compositions

The optimization of the post-column reactor was carried out after establishing the appropriate gradient for the HPLC separation. Alinear gradient from acetonitrile–water [0.06% trifluoroacetic acid (TFA) (2:8) to acetonitrile–water (0.5% TFA) (9:1) within 15 min proved to be sufficient for all the cleavage kinetics investigated.

The initial values of the eluent and reagent compositions and the mixing ratio were taken from test-tube experiments. The reagent concentrations and the flow-rate were varied stepwise until optimum values were found. The flow-rate of the eluent was adjusted to 1 ml min^{-1}. The flow-rates of the pheneanthrenequinone reagent and of the sulphuric acid were initially adjusted to 0.5 ml min^{-1} while that of the sodium hydroxide solution was optimized. Thereafter the flow-rates of the other reagents were subsequently optimized. Fig. 2 shows the relationship between the fluorescent yield and the reagent composition.

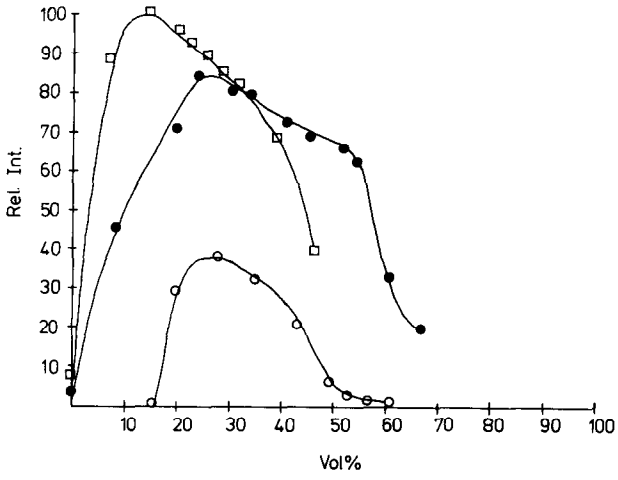

Fig. 2. Dependence of fluorescence yield on reagent composition. Flow-rate of eluent, 1 ml min^{-1}; initial flow-rate of reagents, 0.5 ml min^{-1}; temperature, 95°C (coil I) and ambient (coil II). (○) 0.2 mol l^{-1} NaOH; (●) 2.5 mg l^{-1} 9,10-phenanthrenequinone–ethanol; (□) 5% H_2SO_4.

The appropriate length of the PTFE tubing was determined by cutting pieces successively from over-long tubing. The first reaction step, the formation of 2-(ω-imidoglutamic acid)-1*H*-phenanthro[9,10-*d*]imidazole, under the chosen conditions showed a maximum at a coil length of 16 m, which corresonds to a reaction time of 22 s. The fluorescent dye, 2-amino-1*H*-phenanthro[9,10-*d*]imidazole (API), is formed immediately in a shorter coil only 4 m long, which corresponds to a reaction time of 5 s.

Cleavage conditions

Protected arginine derivatives, *e.g.*, (*Z*)-Arg(Pmc)-OH (5 mmol), were reacted under standard cleavage conditions at 23°C with 1 ml of trifluoracetic acid–thioanisole (4:1). To establish the kinetics, periodically aliquots of 100 μl were drawn and neutralized with 150 μl of triethylamine. Volumes of 20 μl of each of the samples were injected.

RESULTS AND DISCUSSION

All investigations of the cleavage of protecting groups of the guanidino residue in arginine derivatives were made with N-protected arginine derivatives. Such arginine derivatives should be useful models for natural and synthetic peptides. However, as is shown later, the N-α-benzyloxycarbonyl (*Z*) group is also cleaved under the commonly chosen acidic conditions in the presence of thioanisole.

The direct chromatograms (see Fig. 3), which were obtained with UV detection at wavelengths between 200 and 225 nm, show numerous peaks in addition to the expected components, guanidino-protected (*Z*)-α-arginine, free (*Z*)-α-arginine, free arginine and the released protecting groups. The additional peaks are caused

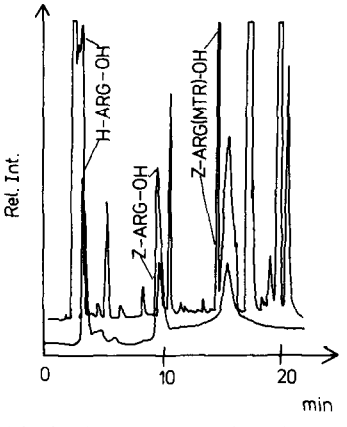

Fig. 3. Chromatogram of (*Z*)-Arg(Mtr)-OH after treatment with TFA–thioanisole for 30 min at 23°C, with UV and fluorescence detection. Column Nucleosil 100-C_{18}, 5 μm (250 × 4 mm I.D.); solvents, (A) acetonitrile–water (0.06% TFA) (2:8) and (B) acetonitrile–water (0.5% TFA) (9:1); gradient, linear from A to B in 15 min; sample volume, 20 μl; direct detection (upper chromatogram), UV, 220 nm; post-column reaction (lower chromatogram); flow-rate of reagent 1 (1 mol l^{-1} NaOH), 0.3 ml min^{-1}; flow-rate of reagent 2 [phenanthrenequinone (2.5 mg l^{-1}) in ethanol], 0.5 ml min^{-1}; flow-rate of reagent 3 (5% H_2SO_4), 0.3 ml min^{-1}; reaction coil 1, 16 m × 0.25 mm I.D., 95°C; reaction coil 2, 4 m × 0.25 mm, ambient temperature; detector, fluorescence, 315/395 nm.

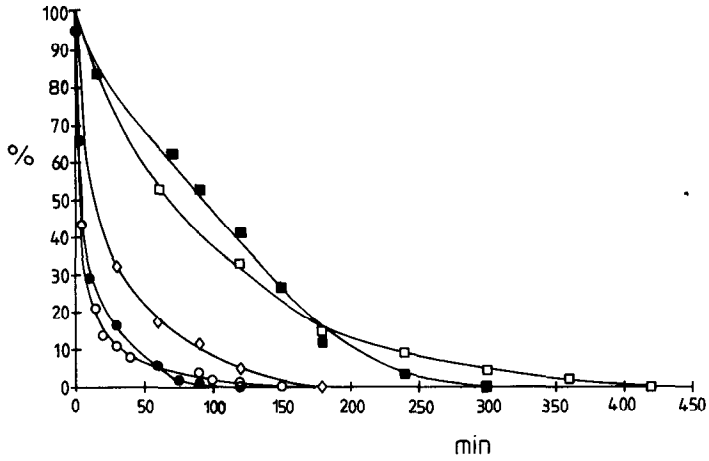

Fig. 4. Decrease in some guanidino-protected arginine derivatives under acidic conditions. Cleavage conditions: 5 mmol of derivative in 1 ml of trifluoroacetic acid–thioanisole (4:1), 23°C. Chromatographic conditions as in Fig. 2. ○ = (*Z*)-Arg(Tip)-OH; ● = (*Z*)-Arg(Pmc)-OH; ◇ = (*Z*)-Arg(Mtr)-OH; □ = (*Z*)-Arg(Mbs)-OH; ■ = (*Z*)-Arg(Mts)-OH.

predominantly by the additon of thioanisole to the acidic reagent and were therefore subsequently not further investigated. The chromatograms resulting from the post-column reactor show a high selectivity for the released arginine derivatives with unprotected guanidino groups. Only one non-specific broad peak was eluted additionally after (*Z*)-Arg(Mtr)-OH, which did not interfere with the interpretation of the chromatograms, however.

The decrease in the fully protected arginine derivatives in the cleavage kinetics of some of the investigated guanidino-protected derivatives is shown in Fig. 4. The reactions are complete within 120 min [(*Z*)-Arg(Pmc)-OH] and 425 min [(*Z*)-Arg(Mtr)-OH]. However, the yields of the expected compounds investigated are in all instances much lower than the theoretical values. Fig. 5 shows, for example, the cleavage kinetics of (*Z*)-Arg(Pmc)-OH. The guanidino-free derivative is released very

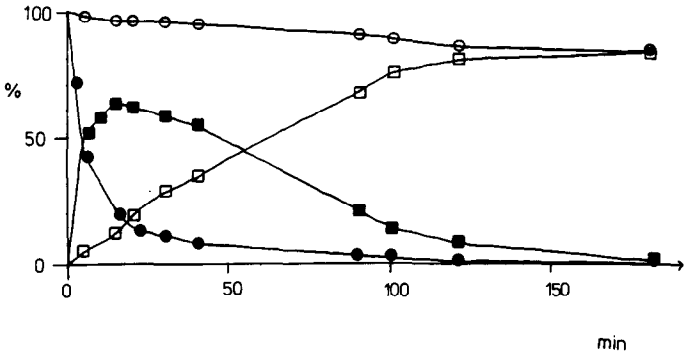

Fig. 5. Cleavage kinetics of (*Z*)-Arg(Pmc)-OH under acidic conditions. Cleavage conditions: 5 mmol (*Z*)-Arg(Pmc)-OH in 1 ml of trifluoroacetic acid–thioanisole (4:1), 23°C. Chromatographic conditions as in Fig. 3. ● = (*Z*)-Arg(PMc)-OH; □ = H-Arg-OH; ■ = (*Z*)-Arg-OH; ○ = sum.

TABLE I

PRODUCT YIELD *VERSUS* REACTION TIME AT REACTION RATES[a] OF 95% AND 99%

Protecting group	*Reaction rate*			
	95%		*99%*	
	Reaction time (min)	*yield (%)*	*Reaction time (min)*	*yield (%)*
(*Z*)-Arg(Tip)-OH	60	78	180	60
(*Z*)-Arg(Pms)-OH	130	88	180	90
(*Z*)-Arg(Mtr)-OH	140	85	175	80
(*Z*)-Arg(Mbs)-OH	225	88	290	85
(*Z*)-Arg(Mts)-OH	310	85	400	82

[a] Decrease in the fully protected arginine derivative. Cleavage conditions: 5 mmol of arginine derivative in 1 ml of trifluoroacetic acid–thioanisole (4:1), 23°C.

fast at the beginning of the reaction. However, after about 10 min the instability of the *Z*-group becomes substantial, and the first formed (*Z*)-Arg-OH is converted to free H-Arg-OH. In Table I the yields and the reaction times for some arginine derivatives are correlated with different reaction rates. The investigated arginine derivatives show different reactivities. The released derivatives are more or less instable and undergo further reactions. Hence the optimum reaction times differ considerably.

In peptide chemistry synthetic strategies have been developed that require the combination of different protecting groups. Therefore, it is very important to correlate these protecting groups according to their reactivity. The investigated derivatives cover a range of optimum reaction times from 60 to 400 min.

The investigations carried out in our laboratory allow the currently most often used protecting groups for the guanidino group in arginine derivatives to be classified according to their reactivity and their overall yield.

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