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Variation in proton affinity of the guanidino group between free and blocked arginine

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Summary. In this paper, the analog of arginine residues in peptides was synthesized and characterized by ESI-MS/MS (electrospray ionization with tandem mass spectrometry), ^{31}P NMR, ^{1}H NMR, IR and high-resolution mass spectrometry. When the Todd reaction activity of the guanidino group in free arginine and the arginine peptide analog were compared, it was found that the proton affinity of the guanidino group was decreased when both the N- and the C-terminal were blocked. As a result, the guanidino group of arginine residues in peptides could be phosphorylated under the Todd reaction condition, but not the free arginine. This result was further proved by the theoretical calculation of their proton affinity.

Keywords: Arginine residues – Guanidino group – Proton affinity – Todd reaction

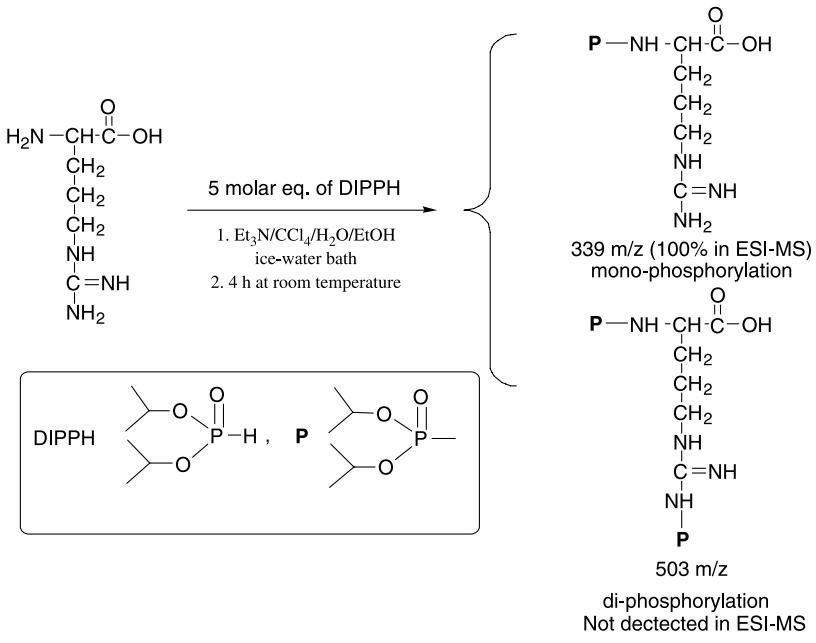
Introduction

O-phosphorylation of proteins plays a vital role in many biological processes, due to its regulative effect on the enzyme activities. It has been reported that phosphorylation and dephosphorylation of proteins (Ju et al., 1995; Zhao et al., 1993) might go through high-coordination phosphoric intermediates for many enzyme catalytic mechanisms (Lee et al., 1996; Bernstein et al., 1997). Nevertheless, N-phosphoryl amino acids and peptides also exhibit some biomimetic reactivity (Xue et al., 1988; Ma et al., 1989), such as peptide formation (Zhang et al., 1994; Li et al., 1992), ester formation (Ma and Zhao, 1992), ester exchange on phosphorus (Li et al., 1993; Ma and Zhao, 1989), and oligonucleotide formation (Zhou et al., 1996). Hence, understanding the intrinsic relationship between the phosphoryl groups and the amino acid residues in peptide is of significance.

The modified Todd reaction has already been successfully applied to the synthesis of N-phosphoryl amino acids (Ji et al., 1988). It was reported that among the twenty natural amino acids, the side-chains of the basic amino acids, such as lysine and histidine, could also be phosphorylated in the modified Todd reaction process (Yin et al., 1993a, b; Li et al., 1993; Zhou et al., 1996). The present paper examines whether this also applies to the other basic amino acid, arginine. In Scheme 1, a CCl_4 solution of diisopropyl phosphite (DIPPH, 15 mmol) was added in drops to a suspension of arginine (3 mmol) in Et_3N (2 ml), H_2O (10 ml) and EtOH (2 ml), followed by vigorous stirring on an ice-water bath. After this addition, the reaction proceeded for 4 h at room temperature, and was then quenched by acidifying the mixture to pH 2–3 with dilute HCl. Then it was extracted with ethyl acetate, dried with anhydrous $MgSO_4$, and analyzed by ESI-MS. The mass spectra (Fig. 1) showed that no di-phosphorylated product was detected even with the presence of excess DIPPH.

The implication of Scheme 1 is that the guanidino group of free arginine could not be phosphorylated under Todd reaction conditions even though arginine was protected on the N-terminal. The guanidino group of arginine with pK_a 12.48 is the strongest basic group among the twenty α-amino acids, and is easily protonated under Todd reaction conditions with pH = 9–9.5. Consequently, it is a poor nucleophile for the phosphorylation reaction.

However, when Growth Hormone Releasing Factor (GRF1-29 amino acids: NH_2- Arg- Ser- Met- Ile- Asp-



Scheme 1. Synthesis pathway of N-diisopropyl-oxyphosphoryl arginine

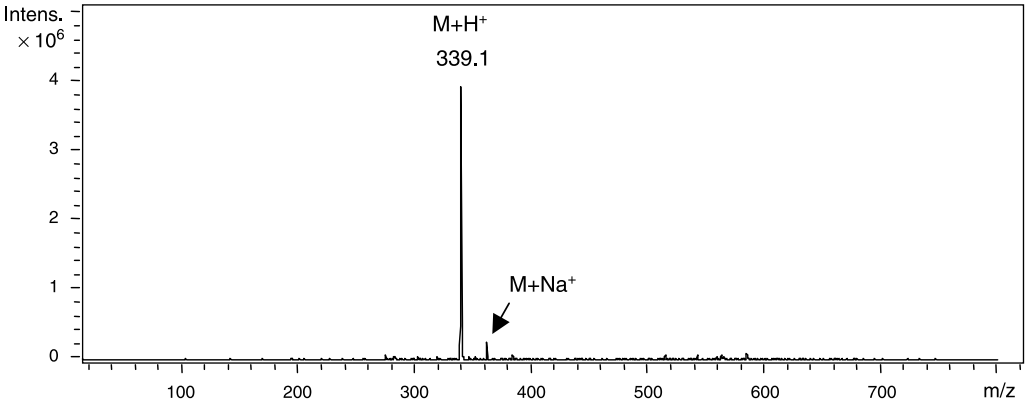


Fig. 1. ESI-MS spectrum of the organic layer in the reaction products from Scheme 1

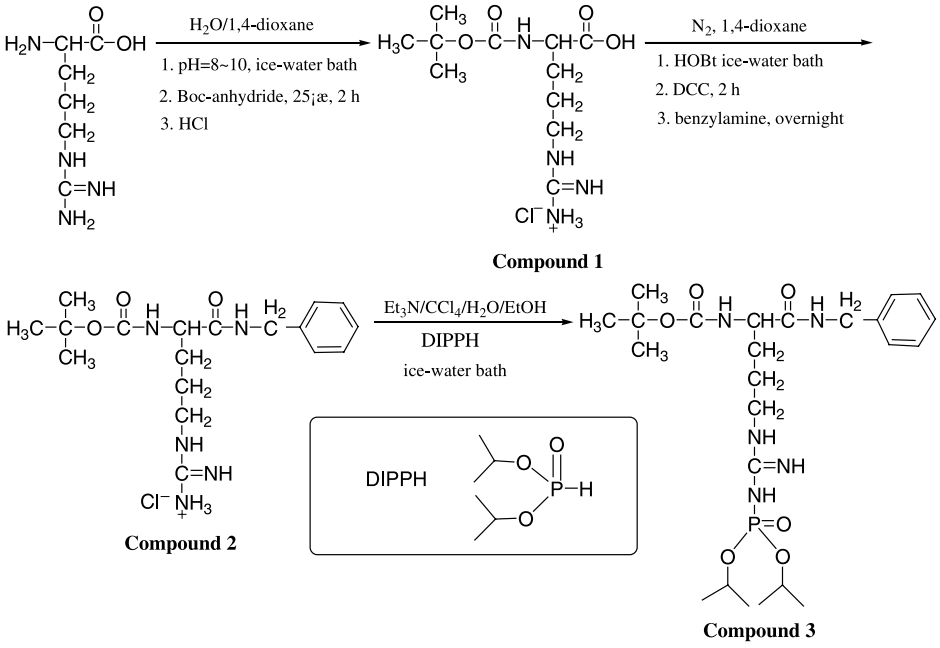
Gln- Leu- Leu- Lys- Arg- Ala- Ser- Leu- Gln- Gly- Leu- Val- Lys- Arg- Tyr- Ser- Asn- Thr- Phe- Ile- Ala- Asp- Ala- Tyr-OH) was reacted with DIPPH under a modified Todd reaction condition, it was worth noting that the arginine residues in the GRF could be phosphorylated (Liu et al., 2005). Thus, the guanidino groups of arginine residues in peptides behaved differently from the free arginine under the Todd reaction condition. To investigate this phenomenon further, in the present paper, the target product compound 3 was designed and prepared in a lower yield (Scheme 2), which was used to simulate the arginine residue in peptide. The structure of compound 3 was characterized through ESI-MS/MS, HRMS and NMR (^{31}P, ^{1}H and ^{13}C).

Materials and methods

Materials and equipment

Arginine, Boc-anhydride (di-tert-butyl dicarbonate), DCC (N′N-carbonyl-diimidazole) and HOBt (1-hydroxybenzotriazole) were purchased from GL Biochem (Shanghai, China) Ltd. Benzylamine was acquired from a local company. Diisopropyloxyphosphite was prepared in our laboratory. All other chemicals were analytical grade.

The mass spectra were obtained using a Bruker ESQUIRE 3000 plus ion trap spectrometer equipped with an ESI ion source. Operating conditions for ESI in the positive ion mode were as follows: spray voltage: 4000 V; capillary temperature: 300 °C; dry gas (N_2): 4 l/min; Nebulizer (N_2): 7 psi. Using a Varian Unity plus 500 MHz NMR spectrometers, ^{31}P, ^{1}H and ^{13}C NMR spectra were recorded. The ^{31}P NMR spectrum was calibrated externally using 85% phosphoric acid as a reference.



Scheme 2. Synthesis pathway of the target compound 3

Synthesis of compounds

(1-Benzylcarbamoyl-4-guanidino-butyl)-carbamic acid tert-butyl ester (Compound 2)

Arginine (10 mmol) was dissolved in 20 ml aqueous solution with a pH of 8–10 by adding 2 mmol/l sodium hydroxide solution (5 ml) on an ice-water bath, and then adding 10 ml 1,4-dioxane. Then 1.1 equivalent of Boc-anhydride (di-tert-butyl dicarbonate, 11 mmol) was added in drops, and stirred at room temperature for two hours. The solvent was then removed under reduced pressure. The resulting solution was acidified to pH 3 with dilute HCl, and the aqueous phase was washed with ethyl acetate (3×20 ml). White crystal slurry was then separated from the aqueous layer after depositing it at room temperature for 4 h. The crystals were filtered, washed and dried in vacuo at room temperature to yield 2.26 g of compound 1 (Boc-Arginine HCl) in 73% yield.

Following this, under the protection of N_2, compound 1 (5 mmol) suspended in 60 ml anhydrous 1,4-dioxane, HOBt (1-hydroxybenzotriazole, 5.5 mmol) was added to the suspension solution on an ice-water bath. DCC (N′N-carbonyldiimidazole, 5.5 mmol) in 9 ml anhydrous 1,4-dioxane was added in drops to the above mixture and stirred for 2 h. Then, benzylamine (5.5 mmol) in 2 ml anhydrous 1,4-dioxane was added to the activation system and stirred overnight. After filtering the white precipitate, the yellowy solvent was evaporated. The residue was purified by column chromatography on silica gel using chloroform/methanol (V/V = 10:1) as an eluent and compound 2 was obtained in 32% yield.

(1-Benzylcarbamoyl-4-[N-diisopropyloxyphosphoryl guanidino]-butyl)-carbamic acid tert-butyl ester (Compound 3)

To the stirred solution of compound 2 (0.275 mmol) in a mixture of water (0.6 ml), EtOH (1 ml) and Et_3N (0.5 ml) on an ice-water bath, a solution of diisopropyloxyphosphite (DIPPH, 0.302 mmol) in CCl_4 (0.5 ml) was added, and then stirred for 4 h at room temperature. The solvent was evaporated and the residue was purified by column chromatography on silica gel using dichloromethanol/cyclohexane/methanol (V/V = 10:1.5:1) as an eluent to give the final product, compound 3, in 12% yield as an oil. Spectral data for compound 3: HRMS: calc. mass for $C_{24}H_{42}N_5O_6P$ $[M+H]^+$: m/z 528.2951. Found: m/z 528.2949. ^{31}P NMR ($CDCl_3$, 202 MHz, ppm): 6.796. ^1H NMR ($CDCl_3$, 500 MHz, ppm): 1.27 (m, 12H, $4CH_3$), 1.41(d, 9H, $3CH_3$), 1.62 (m, 2H, CH_2), 1.85 (m, 2H, CH_2), 3.22 (m, 2H, CH_2), 4.18 (m, 1H, CH–CO), 4.43 (m, 2H, 2CH), 4.53 (m, 2H, CH_2-Ph), 5.75 (br, 3H, 3NH), 7.25 (m, 5H, H_{arom}), 7.43 (br, 1H, NH), 7.86 (br, 1H, NH). IR (NaCl windows): 1242 cm^{-1} (P = O vibration), 1007 cm^{-1} (P–O vibration).

Results and discussion

Characterization of the structure of compound 3 with ESI-MS/MS

It is well known that electrospray ionization with tandem mass spectrometry (ESI-MS/MS) is a very powerful tool for structural elucidation. Therefore, the structure of compound 3 was first analyzed by ESI-MS/MS. The $[M+H^+]$ ion at m/z 528 of compound 3 was activated and dissociated through collision with collision gas (collision induced dissociation, CID) in an ion trap (Fig. 2). Special neutral molecules, such as isobutene, carbon dioxide, propene and benzylamine, were lost from precursor ions to produce a series of fragment ions at m/z 428, 386, 344, 264, 247, 157 and 140. From the cleavage pathway described in Scheme 3, it was observed that the $[M+H]^+$ ion at m/z 528 of compound 3 lost one molecule of isobutene and carbon dioxide to yield the $[M\text{-}56\text{-}44+H]^+$ ion at m/z 428, which indicated that compound 3 contained

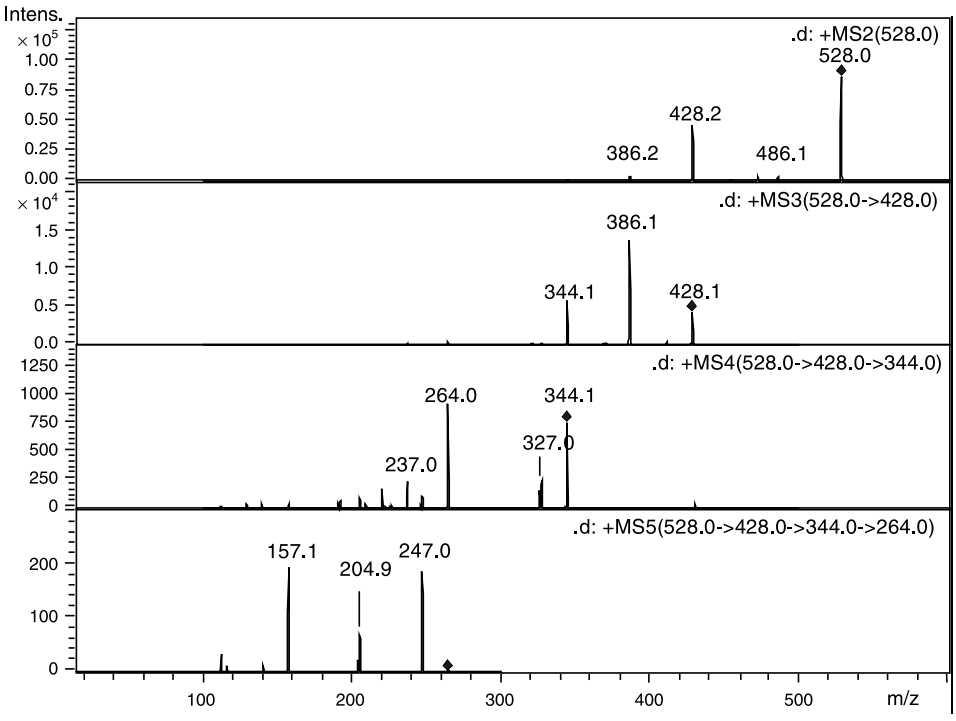


Fig. 2. ESI-MS^n spectra of the compound 3 $[M+H]^+$ ion at m/z 528 ($n=2$–5) in positive mode

Boc-group. The loss of two propene molecules one after the other from the fragment ion at m/z 428 indicated the existence of diisopropyloxyphosphoryl group in the compound (Jiang et al., 2000). Similarly, one benzylamine molecule was lost from the fragment ion at m/z 264 to yield ion m/z 157, from which it is implied that the analyzed compound contained the fragment of benzylamine. According to the above discussion, from the structure of compound 3 it was proved that the side-chain guanidino group of the arginine residues was phosphorylated under Todd reaction conditions. In other words, when both the amino group and the carboxy group of the arginine were fixed, as in compound 2, an analog for peptide, the guanidino group could be phosphorylated.

Calculation of the difference of proton affinity (PA) between free and blocked arginine

For free arginine, the pK_a of the guanidino group is 12.48. Under Todd reaction conditions with a pH of 9–9.5, the guanidino group is predominantly protonated and it could not act as a nucleophile. The question we sought to answer, however, was how compound 2 could be phosphorylated under such conditions. It was deduced that the basicity of the guanidino group in compound 2 changed when both the N- and the C-terminals of the free arginine were blocked. Hence the basicity of a protonable group could be varied as a function of its environment. It is possible to measure the pK_a experimentally, but this is generally not an easy task. In a gas phase environment, the proton affinity (PA) can be used to describe the ability of a molecule to accept a proton. Hence, we used the PA quantity to describe the nucleophilicity difference between guanidino groups containing arginine residues in peptides and those with free arginine.

The proton affinity for the reaction $A + H^+ \rightarrow AH^+$ is defined as the negative of the reaction enthalpy at 298.15 K, and hence:

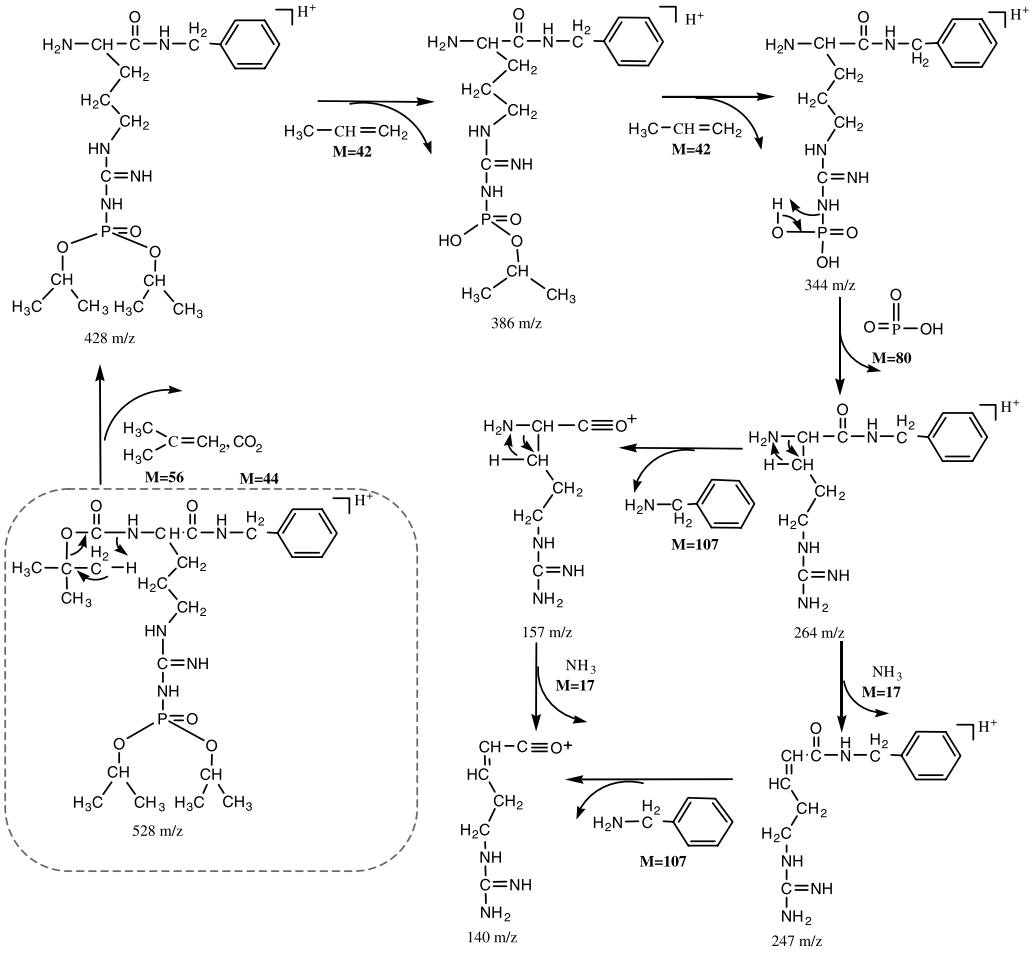
$$PA = -\Delta H = -\Delta E + RT \tag{1}$$

where T = temperature, R = ideal gas constant.

In order to simplify the calculation process, both the N- and the C-terminals of free arginine were blocked with methyl group to simulate the arginine residue in peptide. If only the guanidino groups of the free and blocked arginine are protonated, their PA is described as following Eqs. (2) and (3), respectively:

$$PA(A) = -\Delta E(A) + RT = -[E(AH^+) - E(A)] + RT \tag{2}$$

$$PA(B) = -\Delta E(B) + RT = -[E(BH^+) - E(B)] + RT \tag{3}$$



Scheme 3. ESI mass spectral fragmentation pathway of compound 3 in positive mode

Therefore,

$$\Delta\mathrm{PA} = \mathrm{PA(A)} - \mathrm{PA(B)} = -\Delta E(\mathrm{A}) + RT - (-\Delta E(\mathrm{B}) + RT) = \Delta E(\mathrm{B}) - \Delta E(\mathrm{A}) \quad (4)$$

Arginine, protonated arginine, blocked arginine and its protonated structure were built manually on an SGI workstation, using the Tripos builder module, respectively. Then, their minimum conformations were searched using a random search method. On the basis of their respective minimum conformations, E (arginine), E (protonated-arginine), E (blocked-arginine) and E (protonated blocked-arginine) were calculated at HF/6-31G* level using the GAUSSIAN98 software (Frisch et al., 1998). The corresponding results of $\Delta E(\mathrm{B})$ and $\Delta E(\mathrm{A})$ are shown as follows:

$$\Delta E(\mathrm{B}) = E(\text{protonated blocked arginine, RHF}) - E(\text{blocked arginine, RHF}) = -0.3569 \text{ au}$$

$$\Delta E(\mathrm{A}) = E(\text{protonated arginine, RHF}) - E(\text{arginine, RHF}) = -0.3674 \text{ au}$$

Putting the values of $\Delta E(\mathrm{B})$ and $\Delta E(\mathrm{A})$ into Eq. (4), the value of $\Delta\mathrm{PA}$ is 0.0105 au. If atom energy unity is converted to $\mathrm{kJ \cdot mol^{-1}}$, the value of $\Delta\mathrm{PA}$ is $27.67\ \mathrm{kJ \cdot mol^{-1}}$. The results of this calculation gave us the important information that the basic property of the guanidino group in the arginine residue of peptides is weaker than that of the guanidino group in free arginine.

Conclusion

In conclusion, the experiments described above showed that the chemical environment in peptides or proteins causes variations in the PA of the amino acid side chain. For instance, when arginine residue is situated in peptide, its guanidino group has a weaker PA than free arginine. As a result, the unprotonated guanidino group could be phosphorylated under modified Todd reaction conditions, while the guanidino group of the free arginine has a stronger PA to prevent the phosphorylation. Yollete et al. reported that arginine residues have emerged as general

bases in several enzymes, such as IMP dehydrogenase, pectate/pectin lyases, fumarate reductase, and L-aspartate oxidase (Schlippe and Hedstrom, 2005). It is also indicated that the chemical environment in peptides or proteins has an important effect on the proton affinity of the guanidino group in arginine residues.

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