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On the Electrophilicity of Cyclic Acylphosphoramidates (CAPAs) Postulated as Intermediates

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Cyclic acylphosphoramidates (2-hydroxy-2-oxy-1,3,2-oxazaphospholidine-5-ones, CAPAs) are phosphate-activated amino acids possessing both a carboxyl–phosphoryl anhydride and a phosphoramidate bond in a ring. They structurally resemble NCAs (amino acid *N*-carboxyanhydrides, Leuchs′ anhydrides), which have been extensively investigated. By contrast, the chemistry of CAPAs has remained almost unexplored since it was proposed in a prebiotic reaction of inorganic polyphosphates (PolyPs) with amino acids. In the present work, the bielectrophilicity of α-CAPAs (Gly-CAPA, Ala-CAPA) was identified by isotopic analysis (^{18}O, ^{15}N) and further proved by trapping α-CAPA with nucleophiles such as water, amino acids, phosphate and methanol in alkaline media, which yielded *N*-phosphoamino acids and peptide phosphoanhydride and phosphate ester derivatives. By comparison with the reactivity of NCAs, the bielectrophilicity of CAPAs indicates that CAPAs can provide rich chemistry.

Introduction

α-Amino acid *N*-carboxyanhydrides (Leuchs' anhydrides, NCAs, **4′**, Scheme 1) are cyclic and carbonyl-activated amino acids that were recently shown to be accessible intermediates in plausible prebiotic reactions mediated by carbonyl sulfide (COS)[1] or cyanate.[2] NCAs have been investigated extensively with a 100-year[3] history of success in aspects of stepwise peptide syntheses, ring-opening polymerizations and molecular evolution. Interestingly, cyclic acylphosphoramidates (CAPAs, **4**, Scheme 1) are cyclic and phosphoryl-activated amino acids that were first proposed as intermediates in a prebiotic reaction of inorganic polyphosphates (PolyPs) and amino acids with peptide yields up to 36%.[4] Because CAPAs structurally resemble NCAs, it will be of great significance to compare the intrinsic similarities and differences between the two. Unfortunately, the chemistry of CAPAs has remained relatively unexplored since its discovery.

In addition, because of the prevalence of carboxyl–phosphoryl anhydride containing species in phosphate-transfer reactions catalyzed by related enzymes (e.g., EC 2.7.2 subfamily), compounds such as formyl phosphate,[5] acetyl phosphate,[6] aminoacyl phosphates[7] and aminoacyl adenylates[8] have been extensively explored as model compounds. Meanwhile, the biologically less popular phosphoramidate bond has attracted increased attention[9] since the emergence of histidine kinase research.[10] CAPAs possess both a carboxyl–phosphoryl anhydride moiety and a phosphoramidate bond in a ring. Although intact CAPAs have not been detected in living organisms yet, the study of such bifunctional model compounds may enhance our understanding of biological phosphate activation and transfer. Moreover, it has been suggested that inorganic polyphosphate was an ancient energy carrier preceding ATP,[11] and the earliest organisms may have used PolyPs through the use of PolyP-utilizing enzymes;[12] therefore, PolyPs and amino-acid-induced CAPA might serve as a primitive model and provide clues to the later-developed mechanism of PolyP-utilizing enzymes.

Hence, we initiate the present investigation of CAPAs to get insight into the merits of amino acid and phosphate activations.

Results and Discussion

CAPAs could be produced through the reaction of the prebiotically available[13,14] linear polyphosphates or cyclotriphosphate (P_3m, **1**) with amino acids **2**. Notably, P_3m is the most efficient reagent.[4b] The mechanism involves automatic intramolecular ring closure of *N*-triphospho-α-amino acids **3** followed by release of pyrophosphate $\mathbf{P_2}$ to yield CAPAs (Scheme 1). Interestingly, the corresponding *N*-triphospho-β-amino acids and *N*-triphospho-γ-amino acids

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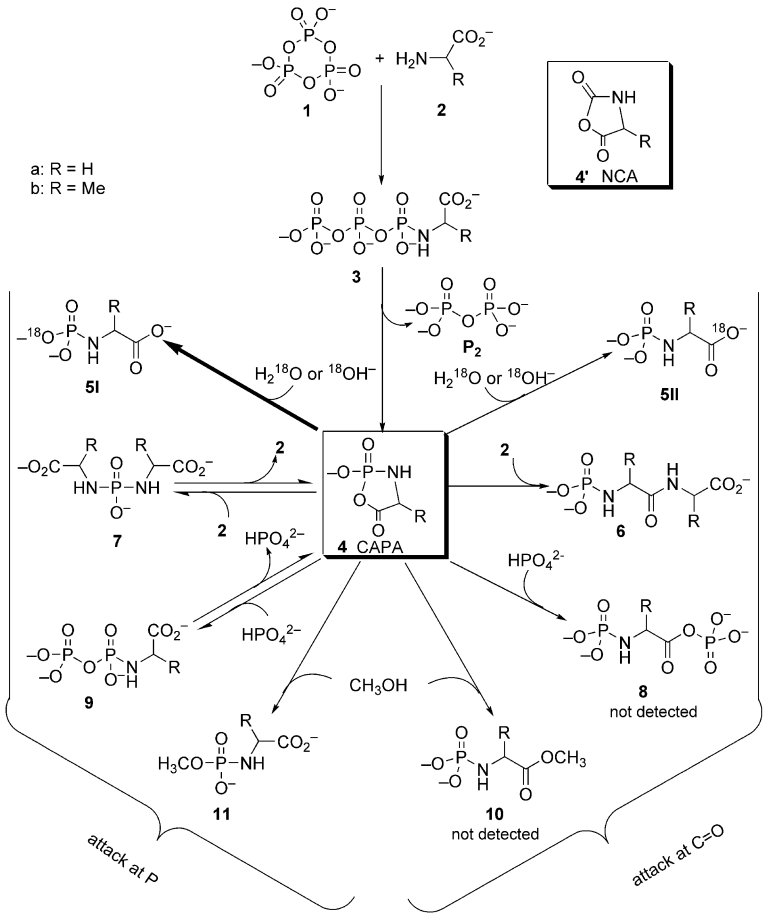


Scheme 1. Bielectrophilicity of CAPAs towards $H_2{}^{18}O$, amino acids, phosphate and methanol.

were fairly stable at room temperature.[15] In the present experiments, the CAPAs were generated in situ by treating **1** with α-amino acids **2**.

Bielectrophilicity of CAPAs towards $H_2{}^{18}O$

N-Phosphoamino acids **5** were detected and proposed as hydrolytic products of CAPAs in previous reports.[4b,4c,16] Such a hydrolysis reaction was recently applied to the synthesis of 13 *N*-phosphoamino acids;[17] however, the detailed hydrolytic mechanism was not confirmed. As with acetyl phosphate[6b,6e] and formyl phosphate,[5c] CAPAs possess a highly reactive carboxyl–phosphoryl mixed anhydride. Depending on the pH, hydrolysis of such anhydrides may undergo bond cleavage in two ways: C–O cleavage or P–O cleavage. In this paper, ^{18}O-isotope analysis was used to determine the cleavage mode in the hydrolysis reaction of CAPAs generated in situ through the reaction of amino acids (Gly or Ala 0.1 M) **2** with **1** (0.1 M) in $H_2{}^{18}O$ (96% ^{18}O) at pH 11.0 and 40 °C. If $H_2{}^{18}O$ attacks the phosphorus centre followed by P–O cleavage, the ^{18}O label should be on the phosphoryl group, leading to product **5I**. If $H_2{}^{18}O$ attacks the carboxyl carbon followed by C–O cleavage, the ^{18}O label should be on the carboxyl group, leading to product **5II**. The structures of *N*-phosphoamino acids **5I/5II** were determined by comparison with authentic samples (Figures S1 and S2, Supporting Information).

A 5:1 ratio of **5I/5II** was observed by ^{31}P NMR spectroscopic analysis of the freshly prepared reaction mixture (Figure 1). The observed upfield isotope shifts (≈0.03 ppm) from **5II** to **5I** as a result of ^{18}O bonded to phosphorus are consistent with literature data.[18] Furthermore, *N*-phosphoglycine **5a** and **5Ia/5IIa** derived from regular H_2O and $H_2{}^{18}O$, respectively, were analyzed by ESI MS–MS. Mass spectra showed a 2 Da unit increment for ^{18}O-incorporated products **5Ia** or **5IIa** (m/z = 178; Figure 2, a and b). The tandem MS^2 fragmentation of **5Ia** and **5IIa** gave the metaphosphate anion $PO_2{}^{18}O^-$ (m/z = 81) and PO_3^- (m/z = 79), respectively, in a 5:1 ratio (Figure 2c), consistent with the ^{31}P NMR spectroscopic result. Hence, NMR spectroscopy and MS show that CAPAs can be attacked by water either at the phosphorus centre or the carbonyl carbon atom under basic conditions. However, attack at phosphorus is dominant for CAPAs, in contrast to the predominant attack at carbonyl centres of acetyl phosphate and formyl phosphate under basic conditions.[5c,6b,6e]

Bielectrophilicity of CAPAs towards the Amino Group

To study the ammonolysis reaction of CAPAs, P_3m (0.1 M) was treated with an excess amount of glycine (1 M)

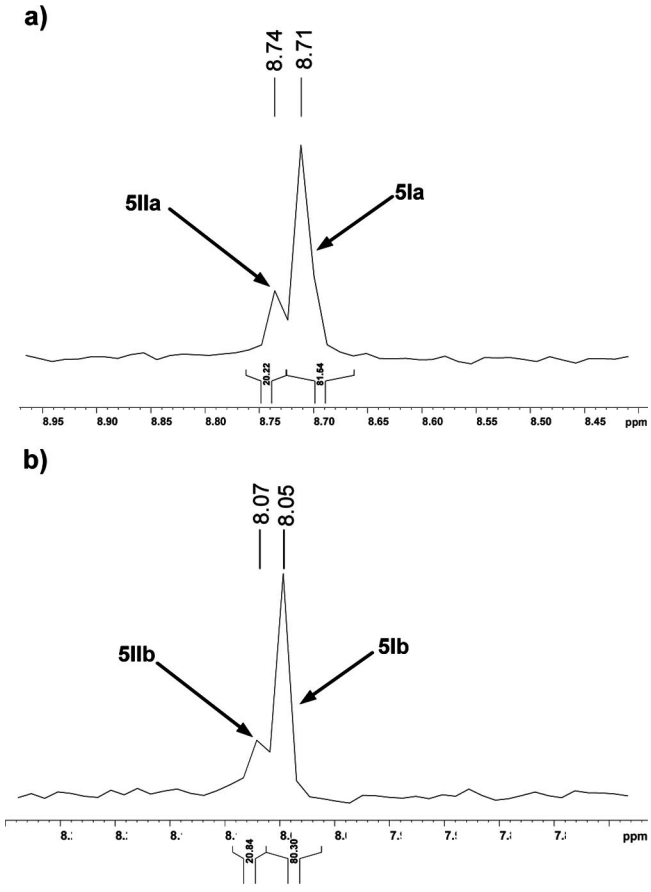


Figure 1. (a) ^{31}P NMR of **5Ia/5IIa** showing a 5:1 ratio. (b) ^{31}P NMR of **5Ib/5IIb** showing a 5:1 ratio.

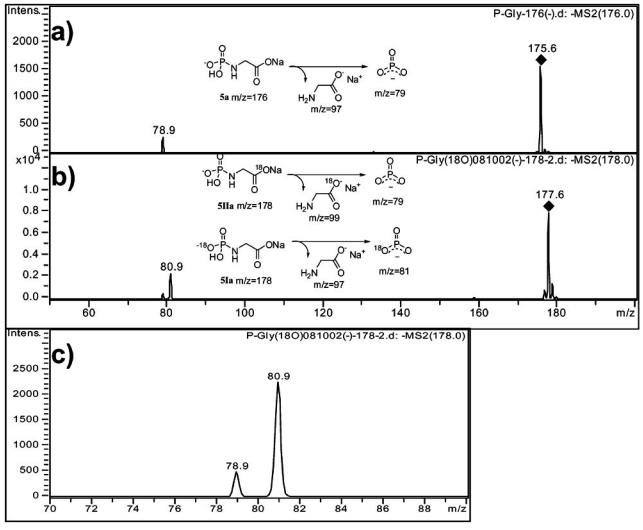


Figure 2. (a) Negative ion ESI MS–MS spectra of authentic *N*-phosphoglycine **5a**. (b) Negative ion ESI MS–MS spectra of ^{18}O-incorporated sample of **5Ia/5IIa**. (c) Magnified spectrum of (b) showing a 5:1 ratio of fragmentation ion (^{18}O-incorporated metaphosphate ion, m/z = 81) from **5Ia** to the fragmentation ion (metaphosphate ion, m/z = 79) from **5IIa**.

at pH 11.0 in water at room temperature and tracked by ^{31}P NMR spectroscopy (Figure 3, a and b). Reactant **1** [δ = 20.70 (s) ppm] was consumed very rapidly. The first intermediate **3a** [δ = –0.51 (d), –4.53 (d), –19.47 ppm (t)] appeared immediately, increased to a maximum after 30 min and then slowly disappeared over 200 min. A second intermediate **7a** [δ = 13.40 (s) ppm] was formed with a maximum concentration of 0.033 M after 40 min and then gradually disappeared after 150 min. Products **5a** [δ = 9.11 (s) ppm] and **6a** [δ = 8.75 (s) ppm] were formed at a later stage and then remained almost constant after 150 min. Byproduct pyrophosphate $\mathbf{P_2}$ [δ = 4.78 (s) ppm] appeared rapidly and increased to a maximum after 200 min.

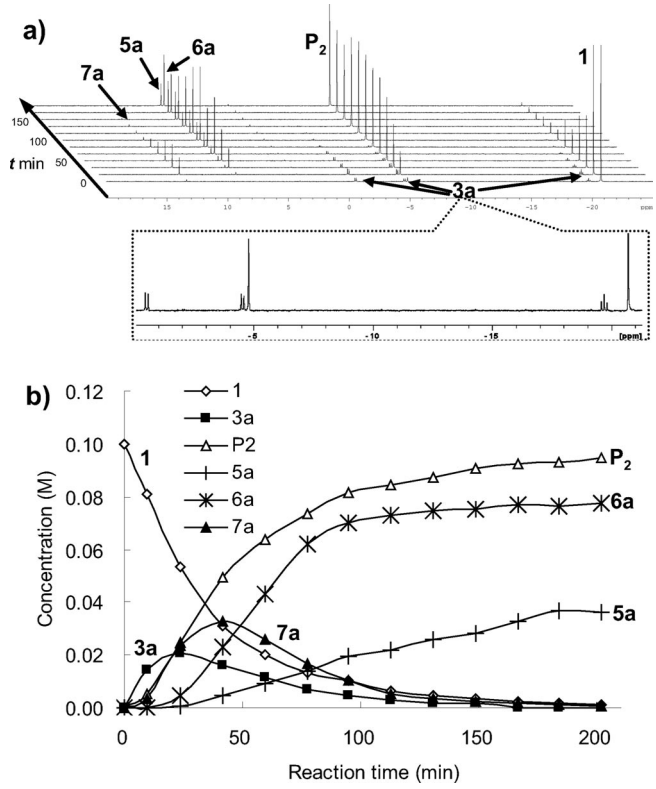


Figure 3. (a) Time-dependent ^{31}P NMR stacking spectra of the reaction of P_3m (0.1 M) with an excess amount of glycine (1 M) at pH 11.0, room temp. (b) Time-dependent concentration curves of reactant, intermediate and products in (a). The concentrations were calculated on the basis of the integration of the ^{31}P NMR peaks.

The observed signal of intermediate *N*-triphosphoglycine **3a** was consistent with previous data.[16] *N*-Phosphoglycylglycine **6a** (yield ≈ 70%) derived from glycine nucleophilic attack at the carbonyl of CAPA was confirmed by comparison with a synthetic sample (Figure S3, Supporting Information). *N*-phosphoglycine **5a** was also identified by comparison with an authentic sample. The remaining significant signal was a decoupled peak for **7a** at δ = 13.40 ppm (singlet), which was transformed into a quintuplet ($J_{H,P}$ = 8.0 Hz) in the ^{1}H-coupled ^{31}P NMR spectrum (Figure 4a), suggesting that there were four equivalent protons from two glycine units to split the phosphorus signal.

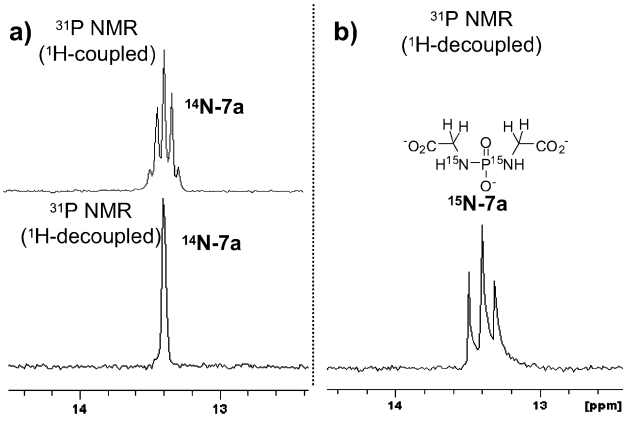


Figure 4. (a) ^{31}P NMR spectrum of **7a** in the reaction of P_3m (0.1 M) with glycine (1 M) at pH 11.0, room temp. (b) ^{31}P NMR spectrum of **7a** in the reaction of P_3m (0.1 M) with ^{15}N-labelled glycine (1 M) at pH 11.0, room temp.

Indeed, the ^{1}H-decoupled ^{31}P NMR spectrum of a reaction mixture derived from ^{15}N-labelled glycine and P_3m showed a triplet ($J_{N,P}$ = 22.4 Hz, close to a reported $J_{N,P}$ = 25.8 Hz[19]) at δ = 13.4 ppm (Figure 4b), indicating that there were two P–N bonds in **7a**. The NMR results support the formation of *N*,*N*′-phosphorylated bisglycine **7a**, which is derived from glycine attack at the phosphorus atom of CAPA. The ^{31}P NMR signal of bisalanine derivative **7b** and *N*-phosphoalanylalanine **6b** were also detected in the reaction of P_3m with alanine (Figure S4, Supporting Information). During the reaction, products **7** gradually disappeared with increasing amounts of products **5** and **6** at the same time, which indicated that products **7** are able to convert back to the intermediate CAPAs **4** through automatic ring closure and release of the amino acids. The hypothesis **7**→**4** is supported by a very close mechanism found in the biological degradation of phosphoric and phosphonic diamide prodrugs.[20] In summary, CAPAs can be attacked by amino acids either at the phosphorus atom or at the carbonyl carbon atom to produce kinetically favourable products **7** and thermodynamically stable products **6**, respectively.

It should be pointed out that previous literature[16] claimed the direct observation of CAPA **4a** by ^{31}P NMR spectroscopy at δ = 13.4 ppm in pH 12 aqueous solution; however, our work shows that CAPAs are too labile to be observed in the presence of nucleophiles such as water and amino acids. In addition, the structural elucidation above and the reaction profile in Figure 3b support the conclusion that the signal near 13.4 ppm in the ^{31}P NMR spectrum comes from the local intermediate *N*,*N*′-phosphorylated bisglycine **7a**, but not the global intermediate CAPA **4a**.

Electrophilicity of CAPAs towards Phosphate and Methanol

Since phosphate activation is important in modern biochemistry, the CAPA generated in situ was treated with phosphate to see whether phosphate would be activated or not. When a mixture of P_3m (0.1 M) and Gly (0.1 M) was reacted in Na_2HPO_4 solution (1 M) at pH 11.0, 40 °C for 12 h, the ^{1}H-decoupled ^{31}P NMR spectrum showed two new peaks with equal intensity at –1.36 and –5.96 ppm, each one a doublet with $J_{P,P}$ = 20.7 Hz (Figure 5b), suggesting that there are two adjacent phosphoryl groups in the new species.

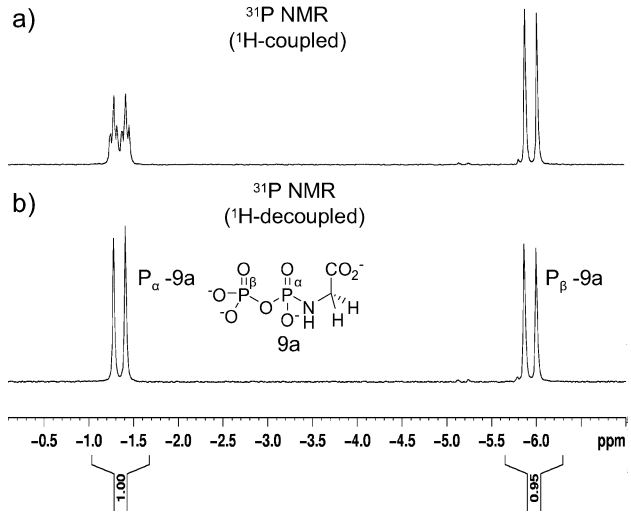


Figure 5. (a) ^{1}H-coupled ^{31}P NMR spectrum of **9a** at pH 11.0. (b) ^{1}H-decoupled ^{31}P NMR spectrum of **9a** at pH 11.0.

In addition, the peaks at –1.36 ppm turned out to be a doublet of triplets ($J_{P,P}$ = 20.7 Hz, $J_{H,P}$ = 6.3 Hz) in the ^{1}H-coupled ^{31}P NMR spectrum (Figure 5a), indicating that this phosphoryl group was adjacent to glycine and also connected to the other phosphoryl group. Consequently, the structure of *N*-pyrophosphoglycine **9a** is proposed. To verify this assignment, **9a** was isolated from the reaction mixture (≈60% conversion yield; ≈30% isolated yield) and its structure was confirmed by ^{1}H, ^{13}C and ^{31}P NMR spectroscopy and HRMS–MS (Figure S5, Supporting Information).

The ^{31}P NMR signal of the *N*-pyrophospho derivatives of other amino acids (such as Ala, Phe, Ser) were also detected in the corresponding reaction mixtures (Figure S6, Supporting Information) but yields were lower because these *N*-pyrophosphoamino acids were less stable and easily decomposed into *N*-phosphoamino acids **5** and inorganic phosphate. Such a situation prevented us from isolating these *N*-pyrophospho derivatives. Hence, we turned to a further reactivity study of stable *N*-pyrophosphoglycine **9a** in hydrolysis experiments at pH 6.8. Time-dependent concentration curves of the hydrolysis products (Figure 6a) revealed that **9a** decomposed into *N*-phosphoglycine **5a** and orthophosphate. Because only trace amounts of $\mathbf{P_2}$ were produced, the mechanism prefers the breaking of the pyrophosphate bond rather than the P–N bond. Together with ^{31}P NMR–pH titration profiles of **9a** (Figure 6b), the hydrolytic mechanism could be rationalized that **9a** was firstly protonated on the β-phosphate, which facilitates the car-

boxyl group to expel the orthophosphate anion with the formation of CAPA **4a**, a path similar to **3a**→**4a**+$\mathbf{P_2}$. Thereby, **5a** is derived from hydrolysis of regenerated CAPA **4a**.

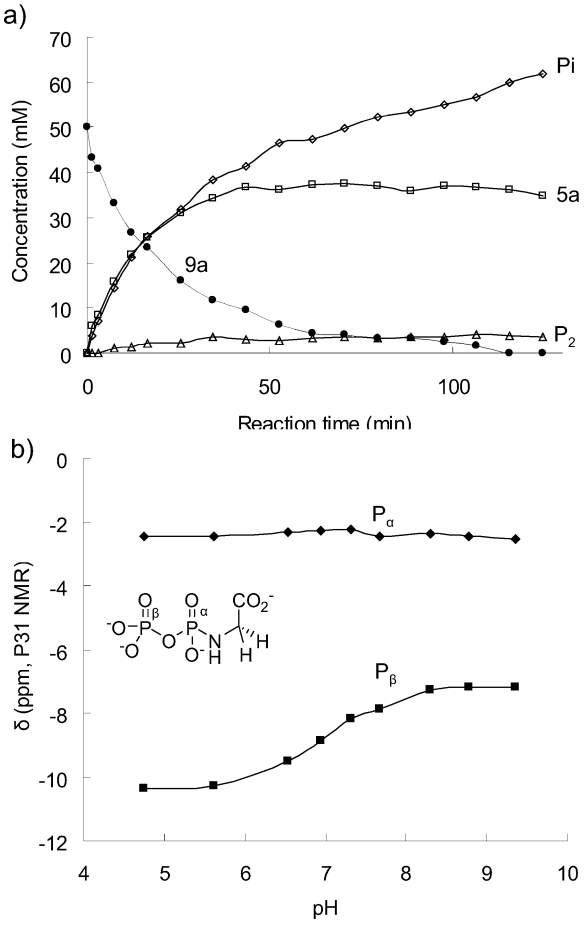


Figure 6. (a) Time-dependent concentration curves of the hydrolysis products of **9a** at pH 6.8 (triethanolamine buffer) 25 °C, $I = 0$. (b) ^{31}P NMR–pH titration profiles of *N*-pyrophosphoglycine **9a**, 25 °C, $I = 0$.

The lability of **9** may be explained by the concept of near attack conformers (NACs) from Bruice.[21] For other amino acids, it could be argued that their side chains provide steric compression, which positions the carboxyl and phosphoryl groups closer, consequently enhancing the intramolecular carboxyl-catalyzed decomposition of **9**. It is noteworthy that a similar mechanism for intermediate **3** should produce the products even faster, as pyrophosphate is a better leaving group than inorganic phosphate.

Indeed, *N*-triphosphoglycine **3a** ($t_{1/2} < 40$ min, pH 11.0, room temp., Figure 3) was less stable than *N*-pyrophosphoglycine **9a** ($t_{1/2}$ = several weeks, pH 11.0, room temp.), which implies that **9** might be a good phosphate donor and energy carrier. In principle, the reaction of CAPAs with phosphate should also produce products **8** derived from attack of phosphate at the carbonyl centre. However, products **8** were not detected in the reaction as a result of the instability of their carboxyl–phosphoryl mixed anhydride bond in basic aqueous solution.

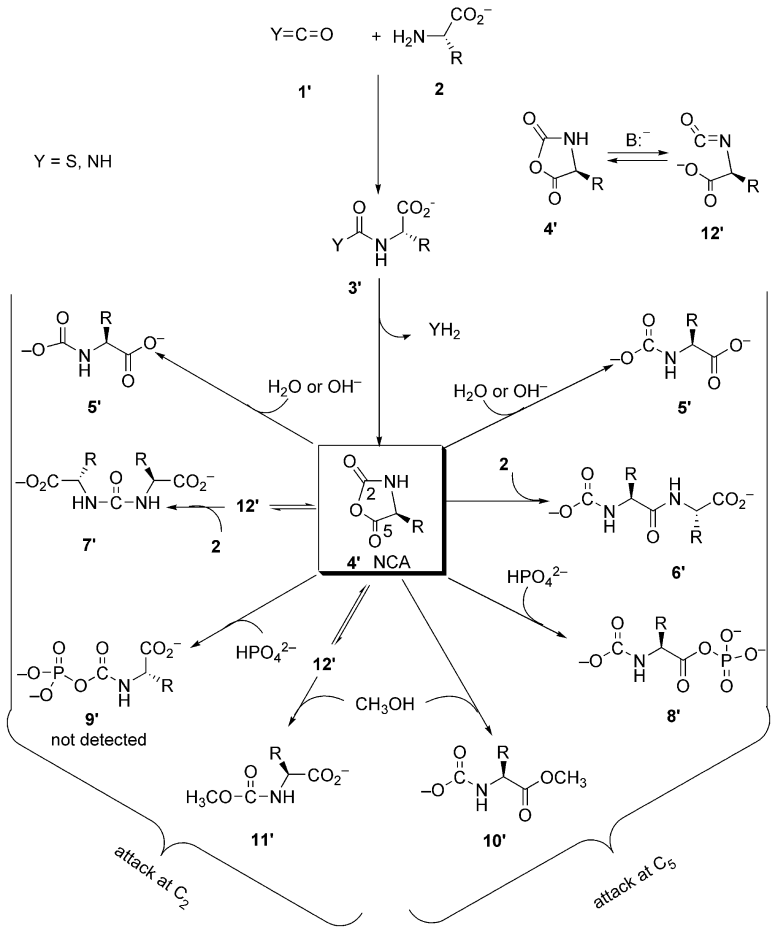
An alkoxide nucleophile was also investigated. When glycine (0.1 M) was treated with P_3m (0.1 M) in methanol/water (1:4) at pH 11.0, 40 °C, 48 h, the corresponding *N*-monomethoxyphosphoglycine **11a** and its hydrolysis product methyl phosphate (MeP) were detected and confirmed by comparing with authentic samples (Figures S7 and S8, Supporting Information). However, product **10** was not detected in the reaction mixture because of the instability of its ester bond in alkaline solution. Concerning the significant role of nucleosides in prebiotic chemistry,[22] a systematic survey of the activation of nucleosides by CAPA is now[23] in progress.

CAPAs vs. NCAs

The formation of and the bielectrophilicity of NCAs toward common nucleophiles such as water, amino acids, phosphate and methanol, which exist in the literature, are compiled in Scheme 2 for comparison with CAPAs. In addition to artificial agents such as phosgene,[24] the formation of NCAs can be achieved by using carbonyl sulfide (COS)[1] and hydrogen cyanate (HNCO).[2] Like pyrophosphate for the formation of CAPAs, H_2S and NH_3 act as good leaving groups to drive the reaction toward NCAs.

Bielectrophilicity

Results of CAPAs suggested that both the phosphorus and carbonyl carbon atoms are possible electrophilic sites and attack at phosphorus is more favourable than attack at the carbonyl carbon. NCAs also possess two electrophilic centres, namely, the carbamoyl group (C-2) and the carbonyl group (C-5). From Scheme 2, C-5 is a preferable reactive site than C-2 towards nucleophiles such as water, amino acids, phosphate and methanol to yield *N*-carboxyl products such as *N*-carboxyl amino acids[25] **5′**, *N*-carboxyl dipeptides[26] **6′**, *N*-carboxyl aminoacyl phosphate[1a,27] **8′** and *N*-carboxyl amino acids ester[28] **10′**. Meanwhile, C-2-directed products such as *N*-alkoxycarbonyl amino acids[29] and *N,N′*-carbonyl bisamino acids[1a,2,30] **7′** were also reported. However, the C-2 related process was proved to be a two-step mechanism involving the formation of isocyanate intermediate[28c,30] **12′** followed by nucleophilic attack of the amino group or methanol at C-2 to obtain the final products. The intrinsic electrophilicity difference between CAPAs and NCAs could be interpreted by the electronic nature of the phosphoramidate group and the carbamoyl group. First, Pauling electronegativity of phosphorus is somewhat lower than that of carbon (2.19 vs. 2.55) when bonded to atoms with larger electronegativity (O, N), and phosphorus retains the larger positive charge, making it more electrophilic. Second, participation of the d orbital may allow the phosphoramidate group to form a possible pentacoordinate intermediate[31] during the nucleophilic reaction. Besides the intrinsic structural factors, solvent effects should also be taken into account. In the present work, the reactions of CAPAs were carried out in water,

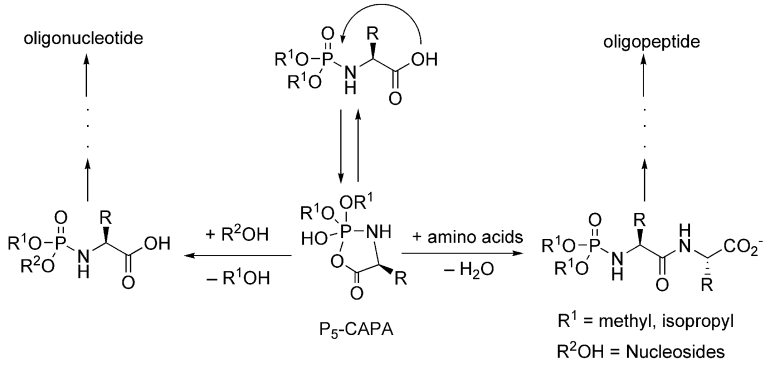


Scheme 2. Formation of NCAs and their bielectrophilicity toward H_2O, amino acids, phosphate and alcohol (data collected from the literature).

which favours systems with large dipole moments like CAPAs, and thus a change in electrophilicity preference might be expected if the reactions are performed in organic solvent.

We previously reported another species,[32] pentacoordinated cyclic acylphosphoramidates (P_5-CAPAs), which also resemble NCAs. They can be formed by automatic ring closure of *N*-dialkyloxyphosphoryl α-amino acids. P_5-CAPAs are bielectrophilically reactive towards amino acids and nucleosides, leading to peptide and nucleic acid oligomerization, respectively (Scheme 3<yschr3 pos="x22">).

The concept that "phosphorus is a carbon copy" is successful in the interpretation of similarity between low-coordinate phosphorus compounds and unsaturated carbon compounds.[33] Apparently, this concept could not be simply extended to high-coordination phosphorus chemistry and related carbon chemistry with the examples of CAPAs and NCAs here.



Scheme 3. Formation and bielectrophilicity of pentacoordinated cyclic acylphosphoramidates (P_5-CAPA).

Peptide Formation, Phosphate Activation and Phosphorylation

The carboxyl group of the amino acid in NCAs is highly activated such that peptide formation can occur at room temperature. The ease of decarboxylation (releasing CO_2 gas) of the initial product *N*-carboxyl peptide makes NCAs prominent in peptide oligomerization and polymerization. When stepwise peptide synthesis is required, *N*-substituted NCAs were developed[3b] because *N*-protecting group forbids the amino group in the decarboxylated product to engage in further coupling and such a *N*-protecting group can be easily removed after the coupling step. Although reactions of amino acids with CAPAs at two electrophilic centres produce *N*,*N*′-phosphorylated bisamino acids **7** besides peptide derivatives **6**. Such a bielectrophilicity will not sacrifice their potential in peptide formation because **7** is thermodynamically unstable and can spontaneously converted back to CAPAs, which again could react with free amino acids to form **6** (Figure 3b). Nevertheless, relative stability of the P–N bond in *N*-phosphoryl peptides under basic condition make CAPAs a potential candidate in stepwise peptide synthesis rather than ring-opening polymerization.

Another interesting aspect of NCAs is phosphate activation related to prebiotic evolution. Reaction of NCAs with inorganic phosphate (Scheme 2) produces aminoacyl phosphates via the precursor *N*-carboxyl aminoacyl phosphates **8′**.[1a,27] In the present work, reaction of CAPAs with inorganic phosphate leads to pyrophosphate derivatives **9**, which are another form of activated phosphate (Scheme 1). Finally, phosphorus is a reactive centre toward several nucleophiles, especially alkoxide nucleophiles, and this makes CAPAs a promising phosphorylating agent having an amino acid conjugate. This may provide an alternative route to the synthesis of phosphoramidate prodrugs of nucleoside analogues.[34] Although alcohol phosphorylation (e.g., nucleosides) by CAPAs in water is not efficient, the problem could be circumvented by transfer of the reaction into anhydrous solvents.

On the basis of the above discussion, CAPAs are not only phosphate-activated amino acids but also amino-acid-activated phosphates as unique phosphoryl donors. Consequently, CAPAs might be considered as potential models that can be used to understand enzymatic phosphoryl transfer mechanisms and aminoacyl transfer processes.

Conclusions

The results described herein indicate that α-CAPA generated in situ from α-amino acids and P_3m is a reactive intermediate in alkaline media. The bielectrophilicity of α-CAPAs (Gly-CAPA, Ala-CAPA) was identified by isotopic analysis (^{18}O, ^{15}N) and further proved by trapping the α-CAPAs with nucleophiles such as water, amino acids, phosphate and methanol in alkaline media, which yielded interesting phosphorylated products. By comparison with the reactivity of NCAs, the bielectrophilicity of CAPAs indicates that they are not only phosphate-activated amino acids but also amino-acid-activated phosphates. Thus, they have the potential to be used as self-protected and self-activated blocks in peptide coupling reactions. Furthermore, the formation process and the reactivity of CAPAs in phosphoryl transfer may have potential biological significance in connection with PolyP-utilizing enzymes, which use PolyPs as phosphoryl and energy sources. Finally, the corresponding phosphorylated products were not observed for the analogous β- and γ-amino acid CAPAs at moderate temperature (data not shown), which thus highlights the unique role of α-amino acids in potential prebiotic evolutionary events.[35]

However, a limitation to the extensive application of CAPAs is the lack of an efficient preparation method of CAPAs. Hence, great effort should be focused on the preparation of pure CAPAs in the future, even though the preparation of Gly-CAPA has been described.[36] It is noteworthy that the reversible conversion between Gly-CAPA **4a** and *N*-pyrophosphoglycine **9a**, demonstrated in this work, may provide an alternative path to the in situ generation of CAPAs, which makes investigations of CAPAs feasible in neutral (Figure 6a) or acidic solutions. Because the development of the chemistry of NCAs is more extensive than that of CAPAs, we believe that successful experiences on NCAs can serve as a reference for the chemistry of CAPAs.

Experimental Section

General: Reagents were purchased from various commercial sources. ^{15}N-glycine from Cambridge Isotope lab and $H_2{}^{18}O$ Shanghai Research Institute of Chemical Industry; ^1H, ^{13}C and ^{31}P NMR spectra were recorded with a Bruker 400 MHz NMR spectrometer. ^1H NMR chemical shifts were measured relative to D_2O (δ = 4.70 ppm). ^{13}C NMR chemical shifts were measured relative to CD_3OD (δ = 49.5 ppm). ^{31}P NMR chemical shifts in D_2O were externally referenced to 85% H_3PO_4 (δ = 0.0 ppm). Chemical shifts are given in ppm and signals are reported as s (singlet), d (doublet), t (triplet), q (quartet), quint. (quintuplet) and m (multiplet). MS (ESI) spectra were acquired with a Bruker Dalton Esquire 3000 plus ion-trap mass spectrometer (Bruker-Daltonik Co., Bremen, Germany). High-resolution mass spectra were performed with an ESI-Q-TOF-MS spectrometer (Micromass, England) and LC–MS-IT-TOF (Shimadzu, Japan).

Reaction of Gly, Ala with P_3m in $H_2{}^{18}O$: Amino acids (0.05 mmol) and P_3m (0.05 mmol) were dissolved in $H_2{}^{18}O$ ([^{18}O]>96%, 0.5 mL) and heated to 40 °C. The pH of the solution was maintained at 11.0 by the frequent addition of $Na^{18}OH$ solution. The reaction was quenched by $Na^{18}OH$ solution after 24 h, and the quenched reaction mixture was mixed with D_2O (0.05 mL, for locking and shimming in NMR) and tested by NMR spectroscopy.

Reaction of an Excess Amount of Gly, Ala with P_3m in Water: Amino acids (4 mmol) and P_3m (0.4 mmol) were dissolved in water (4 mL) at room temperature. The pH of the solution was maintained at 11.0 by adding NaOH solution. The reaction mixture was then monitored by ^{31}P NMR spectroscopy.

Reaction of Amino Acids with P_3m in Na_2HPO_4 Buffer: Amino acids (2 mmol), P_3m (2 mmol) and $Na_2HPO_4{\cdot}10H_2O$ (20 mmol) were dissolved in water (20 mL) at 40 °C. The pH of the solution was

maintained at 11.0 by adding NaOH solution. The reaction mixture was then test by ^{31}P NMR spectroscopy after 12 h.

Isolation of *N*-Pyrophosphoglycine 9a: ^{31}P NMR spectroscopy was used to monitor the following isolation process. The reaction above was quenched after 24 h by adding NaOH (20 mmol), and the solution was slowly cooled to 4 °C. The precipitate (inorganic phosphate) was filtered off. The filtrate was mixed with methanol (20 mL) and then stored at –20 °C for 10 h to precipitate residual inorganic phosphate, pyrophosphate and triphosphate, which were filtered off. The filtrate was then condensed to a volume of 2 mL, to which methanol (4 mL) was slowly added to precipitate the oily crude product **9a**. The oily product was separated and dissolved in 0.1 M NaOH (2 mL) and again precipitated by adding methanol (4 mL, repeat this step 10 times to remove the *N*-phosphoglycine and unreacted glycine). Yield 32%, colourless oil. ^1H NMR (400 MHz, D_2O): δ = 3.43 (d, J = 6.6 Hz, 2 H) ppm. ^{13}C NMR (100 MHz, D_2O): δ = 45.7, 179.8 (d, J = 13.0 Hz) ppm. ^{31}P NMR (162 MHz, D_2O): δ = –6.04 (d, J = 21.5 Hz, 1 P), –1.54 (d, J = 21.5 Hz, 1 P) ppm. LC–HRMS (IT-TOF–): calcd. for $C_2H_6NO_8P_2^-$ 233.9574; found 233.9559. LC–HRMS (IT-TOF–): calcd. for $C_2H_5NNaO_8P_2^-$ 255.9394; found 255.9390. LC–HRMS (IT-TOF–): calcd. for $C_2H_4NNa_2O_8P_2^-$ 277.9213; found 277.9199. Note: no attempt was made to maximize the yield. Attempts to evaporate oily product **9a** to dryness under reduced pressure at 20 °C resulted in decomposition; a small portion of the oily product was weighed and the solvents were evaporated to dryness under 120 °C and the yield was estimated on the basis of the weight of dried material.

Reaction of Gly with P_3m in Methanol/Water: Glycine (0.4 mmol) and P_3m (0.4 mmol) were dissolved in methanol/water (1:4, 4 mL) at 40 °C. The pH of the solution was maintained at 11.0 by adding NaOH solution. The reaction mixture was then monitored by ^{31}P NMR spectroscopy.

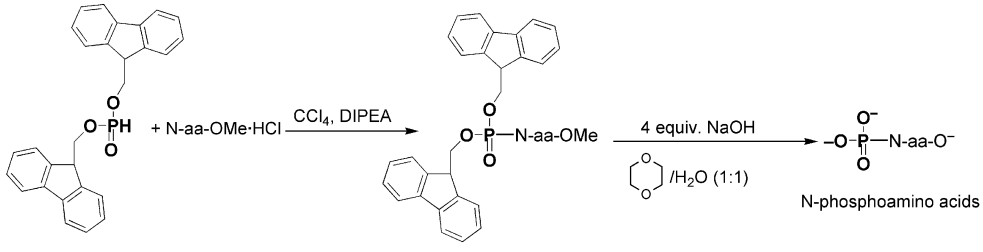
General Procedures for the Synthesis of the Sodium Salt of *N*-Phosphoamino acids: Sodium salts of *N*-phosphoamino acids were synthesized according to a literature method[37] with minor modifications (Scheme 4). The sodium salt of the *N*-phosphoamino acids was obtained through deprotection of the corresponding *N*-bis(9-uorenylmethyl)phosphoryl amino acid methyl esters in dioxane/H_2O (1:1) containing NaOH (4 equiv.).

Bis(9-uorenylmethyl) Phosphite: To a stirred solution of diisopropyl phosphoramidous dichloride (24.74 g, 122.5 mmol) in dichloromethane (200 mL) was added a solution of 9-uorenylmethanol (30.6 g, 196 mmol) in dichloromethane (300 mL) under an atmosphere of argon at 0 °C. The reaction mixture was then stirred for 3 h at room temperature. The solvent was evaporated under reduced pressure to give a viscous residue, which was subsequently dissolved in acetonitrile (250 mL) and chilled to 0 °C. A solution of acetonitrile (50 mL), 1-*H*-tetrazole (5.35 g, 76.4 mmol) and distilled water (20 mL) was then added, and the reaction mixture was stirred overnight. The solution was then diluted in ethyl acetate (400 mL) and sequentially washed with 10% HCl (3 × 90 mL), 10% Na_2CO_3 (150 mL), distilled water (150 mL) and brine (2 × 100 mL). After drying the organic layer with $MgSO_4$, the solvent was removed in vacuo to give a yellow oil. The crude product was puried by crystallization from ethyl acetate to give colourless tiny crystals (28 g, 52%). M.p. 102.2–103.8 °C. ^1H NMR (400 MHz, $CDCl_3$): δ = 4.11 (t, J = 6.5 Hz, 2 H), 4.21–4.31 (m, 4 H), 6.66 (d, J = 706 Hz, 1 H), 7.23–7.30 (m, 4 H), 7.32–7.41 (m, 4 H), 7.47–7.53 (m, 4 H), 7.69–7.73 (m, 4 H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 47.9 (d, J = 6.7 Hz) 67.0 (d, J = 6.3 Hz), 120.0 (d, J = 4.5 Hz), 125.0, 127.1 (d, J = 2.2 Hz), 127.9 (d, J = 2.5 Hz), 141.3, 142.9 (d, J = 9.2 Hz) ppm. ^{31}P NMR (162 MHz, $CDCl_3$): δ = 8.43 ppm.

***N*-Bis(9-uorenylmethyl)phosphoryl Glycine Methyl Ester:** To a stirred solution of glycine methyl ester (1.1 mmol) in acetonitrile (5 mL) and *N*,*N*-diisopropylethylamine (392 µL, 2.2 mmol) was added a solution of bis(9-uorenylmethyl) phosphite (1 mmol) in acetonitrile (1 mL) and carbon tetrachloride (5 mL) by syringe under an atmosphere of argon at 0 °C over 5°min. The reaction mixture was stirred overnight at room temperature. The reaction mixture was then diluted with ethyl acetate (50 mL) and sequentially washed with 10% HCl (50 mL), 10% $NaHCO_3$ (50 mL), distilled water (50 mL) and brine (50 mL). After drying the organic layer with $MgSO_4$, the solvent was removed in vacuo to give a light yellow raw product, which was crystallized from dichloromethane (10 mL) and hexane (35 mL) to give colourless tiny crystals (79%). M.p. 130.6–130.9 °C. ^1H NMR (400 MHz, $CDCl_3$): δ = 3.14 (dt, J = 10.8, 6.4 Hz, 1 H), 3.45 (dd, J = 10.4, 6.4 Hz, 2 H), 3.66 (s, 3 H), 4.13 (t, J = 6.6 Hz, 2 H), 4.20–4.34 (m, 4 H), 7.23–7.30 (m, 4 H), 7.33–7.41 (m, 4 H), 7.48–7.54 (m, 4 H), 7.70–7.74 (m, 4 H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 42.4, 47.9 (d, J = 8.0 Hz), 52.3, 68.0 (d, J = 5.4 Hz), 119.9 (d, J = 5.7 Hz), 125.0 (d, J = 13.6 Hz), 127.0 (d, J = 3.1 Hz), 127.8 (d, J = 5.1 Hz), 141.3 (d, J = 8.0 Hz), 143.3 (d, J = 12.8 Hz), 171.1 (d, J = 7.5 Hz) ppm. ^{31}P NMR (162 MHz, $CDCl_3$): δ = 8.65 ppm. HRMS (ESI-Q-TOF+): calcd. for $C_{31}H_{29}NO_5P^+$ 526.1778; found 526.1793.

***N*-Bis(9-uorenylmethyl)phosphoryl Alanine Methyl Ester:** Title compound was synthesized from (L)-alanine by using the same synthetic procedure of the glycine derivative. Colourless tiny crystals. Yield: 84%. $[\alpha]_D^{20}$ = –5.8 (c = 0.01, $CHCl_3$). M.p. 141.8–142.2 °C. ^1H NMR (400 MHz, $CDCl_3$): δ = 1.20 (d, J = 7.1 Hz, 3 H), 3.25 (t, J = 10.2 Hz, 1 H), 3.60 (s, 3 H), 3.68–3.78 (m, 1 H), 4.10–4.35 (m, 6 H), 7.23–7.31 (m, 4 H), 7.33–7.41 (m, 4 H), 7.47–7.56 (m, 4 H), 7.70–7.74 (m, 4 H) ppm. ^{13} C NMR (100 MHz, $CDCl_3$): δ = 20.9 (d, J = 4.9 Hz), 47.9 (dd, J = 4.1 Hz), 49.8, 52.3, 68.0 (d, J = 5.6 Hz), 119.9 (m), 125.0 (m), 127.0, 127.8 (m), 141.3 (m), 143.3 (m), 174.1 (d, J = 7.1 Hz) ppm. ^{31} P NMR (162 MHz, $CDCl_3$): δ = 7.97 ppm. HRMS (ESI-Q-TOF+): calcd. for $C_{32}H_{31}NO_5P^+$ 540.1934; found 540.1939.

Sodium Salt of *N*-Phosphoglycine (5a): To a stirred solution of bis(9-uorenylmethyl)phosphoryl glycine (249 mg, 0.5 mmol) in di-



Scheme 4. Synthetic path of *N*-phosphoamino acids.

oxane/H_2O (1:1, 10 mL), NaOH (2 mmol) was added. The reaction mixture was stirred overnight at room temperature. The reaction solution was evaporated to a volume of about 1 mL under reduced pressure and mixed with water (4 mL), the solids were ltered out and the ltrate was evaporated to dryness under reduced pressure to give the sodium salt product. White solid, m.p. >300 °C.[1] H NMR (400 MHz, D_2O): δ = 3.23 (d, J = 6.1 Hz, 2 H) ppm. ^{13}C NMR (100 MHz, D_2O): δ = 46.28, 180.1 (d, J = 12.5 Hz) ppm. ^{31}P NMR (162 MHz, D_2O): δ = 8.38 ppm. MS (ESI): m/z = 221.8 $[M + H]^+$.

Sodium Salt of *N*-Phosphoalanine (5b): Title compound was synthesized by using the same synthetic procedure used for **5a**. White solid, m.p. >300 °C. $[\alpha]_D^{20}$ = –2.7 (c = 0.01, H_2O). ^1H NMR (400 MHz, D_2O): δ = 1.18 (d, J = 7.0 Hz, 3 H), 3.42–3.50 (m, 1 H) ppm. ^{13}C NMR (100 MHz, D_2O): δ = 21.6 (d, J = 3.5 Hz), 52.8, 184. 1 (d, J = 8.6 Hz) ppm. ^{31}P NMR (162 MHz, D_2O): δ = 8.07 ppm. MS (ESI): m/z = 235.8 $[M + H]^+$.

General Procedures for the Synthesis of the Lithium Salt of *N*-Phosphoglycylglycine (6a): Title compound was synthesized according to a literature method[37] with minor modification, as shown in Scheme 4. Because the amide bond is labile in the presence of strong bases such as NaOH, **6a** was obtained through deprotection of the corresponding *N*-bis(9-uorenylmethyl)phosphoryl glycylglycine methyl ester in dioxane/H_2O (1:1) containing LiOH (4 equiv.). Detailed synthetic procedures were similar to that of *N*-phosphoamino acids except NaOH was replaced by LiOH in the deprotection step.

***N*-Bis(9-uorenylmethyl)phosphoryl Glycylglycine Methyl Ester:** Yield: 87%, white solid, m.p. 139.6–139.9 °C. ^1H NMR (400 MHz, $CDCl_3$): δ = 3.28 (dd, J = 11.9, 7.0 Hz, 2 H), 3.39 (dt, J = 10.8, 7.4 Hz, 1 H), 3.66 (s, 3 H), 3.86 (d, J = 5.4 Hz, 2 H), 4.10 (t, J = 6.1 Hz, 2 H), 4.23–4.34 (m, 4 H), 6.73 (t, J = 5.3 Hz, 1 H), 7.21–7.29 (m, 4 H), 7.32–7.40 (m, 4 H), 7.43–7.52 (m, 4 H), 7.68–7.73 (m, 4 H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 40.9, 44.2, 48.0 (d, J = 7.8 Hz), 52.3, 68.1 (d, J = 5.6 Hz), 120.0 (d, J = 4.5 Hz), 124.9 (d, J = 8.7 Hz), 127.1, 127.8 (d, J = 5.3 Hz), 141.4 (d, J = 4.8 Hz), 143.2 (d, J = 6.0 Hz), 168.4, 169.9 (d, J = 6.1 Hz) ppm. ^{31}P NMR (162 MHz, $CDCl_3$): δ = 7.62 ppm. HRMS (ESI-Q-TOF+): calcd. for $C_{33}H_3N_2NaO_6P^+$ 605.1812; found 605.1810.

***N*-Phosphoglycylglycine Lithium Salt (6a):** White solid. ^1H NMR (400 MHz, D_2O): δ = 3.40 (d, J = 9.5 Hz, 2 H), 3.71 (s, 2 H) ppm. ^{13}C NMR (100 MHz, D_2O): δ = 43.2, 45.6, 176.4 (d, J = 8.0 Hz), 176.9 ppm. ^{31}P NMR (162 MHz, D_2O): δ = 10.48 ppm. HRMS (ESI-Q-TOF–): calcd. for $C_4H_8N_2O_6P^-$ 211.0125; found 211.0120. HRMS (ESI-Q-TOF–): calcd. for $C_4H_7LiN_2O_6P^-$ 217.0207; found 217.0191.

***N*-(Methoxyphenoxyphosphanyl)glycine Methyl Ester:** Title compound was synthesized according to a literature method.[38,39] Diphenyl phosphite (2 mmol) and a catalytic amounts of Et_3N (about 5 drops) were dissolved in dry THF (5 mL) and cooled to –5 °C. Under an argon atmosphere, CH_3OH (1 mmol) in dry THF (5 mL) was added dropwise to the solution. The solution was stirred for about 30 min and warmed from –5 °C to room temperature during that time. The progress of the reaction was monitored by ^{31}P NMR spectroscopy. At 0 °C, glycine methyl ester hydrochloride (2 mmol), Et_3N (TEA; 0.31 g, 3 mmol) and CCl_4 (3.6 mmol) in CH_2Cl_2 (5 mL) were sequentially added to the resulting solution, and the solution was stirred for about 2 h. The solvent was removed under reduced pressure, and the remaining residue was purified by silica gel column chromatography (ethyl acetate/petroleum ether, 3:1) to afford the target product as a colourless oil (56%). ^1H NMR (400 MHz, $CDCl_3$): δ = 3.14 (dt, J = 11.2, 5.9 Hz, 1 H), 3.74 (s, 3 H), 3.77–3.81 (m, 2 H), 3.83 (d, J = 11.4 Hz, 3 H), 7.14–7.35 (m, 5 H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 42.9, 52.3, 53.7 (d, J = 5.6 Hz), 120.1 (d, J = 5.9 Hz), 124.9, 129.7, 150.7 (d, J = 6.6 Hz), 170.9 (d, J = 8.5 Hz) ppm. ^{31}P NMR (162 MHz, $CDCl_3$): δ = 5.38 ppm. HRMS (ESI-Q-TOF+): calcd. for $C_{10}H_{15}NO_5P^+$ 260.0682; found 260.0696. HRMS (ESI-Q-TOF+): calcd. for $C_{10}H_{14}NaNO_5P^+$ 282.0502; found 282.0500.

Sodium Salt of *N*-Monomethoxyphosphoryl Glycine (11a): Title compound was synthesized according to a literature method.[39] A solution (2 mL) of 0.8 M NaOH (MeOH/H_2O, 1:1) was added to *N*-(methoxyphenoxyphosphanyl)glycine methyl ester (0.2 mmol) and a deprotection reaction was carried out at 60 °C while stirring under an atmosphere of argon for 12 h. The course of the reaction was monitored by ^{31}P NMR spectroscopy until the disappearance of the starting material. Once *N*-(methoxyphenoxyphosphinyl)glycine methyl ester disappeared, the solvent was removed under reduced pressure to give an oily crude product, which was recrystallized from 95% ethanol (1 mL). The precipitate was evaporated to dryness to give the product as a white solid (88%). ^1H NMR (400 MHz, D_2O): δ = 3.31 (d, J = 8.2 Hz, 2 H), 3.43 (d, J = 10.9 Hz, 3 H) ppm. ^{13}C NMR (100 MHz, D_2O): δ = 45.2, 52.0 (d, J = 5.3 Hz), 179.2 (d, J = 9.3 Hz) ppm. ^{31}P NMR (162 MHz, D_2O): δ = 11.88 ppm. LC–HRMS (IT-TOF–): calcd. for $C_3H_7NO_5P^-$ 168.0067; found 168.0081. LC–HRMS (IT-TOF–): calcd. for $C_3H_6NNaO_5P^-$ 189.9887; found 189.9889.

Methyl Phosphate Disodium Salt (MeP): Methyl phosphate was synthesized according to the literature.[40] White solid. ^1H NMR (400 MHz, D_2O): δ = 3.36 (d, J = 10.0 Hz, 3 H) ppm. ^{31}P NMR (162 MHz, D_2O): δ = 8.59 ppm.

Supporting Information (see also the footnote on the first page of this article): Comparison of the ^1H NMR spectra of **5a** and **5b**; ^{31}P NMR spiking spectra of **6a**, **9a**, **11a**, MeP; ^{31}P NMR spectra of **7b** and *N*-pyrophosphorylated derivatives of Ala, Ser, Phe; HRMS–MS spectra of **9a**; ^{31}P, ^1H and ^{13}C NMR spectra of **6a** and **9a**.

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