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Traceless Staudinger Ligation to Access Stable Aminoacyl- or Peptidyl-Dinucleotide

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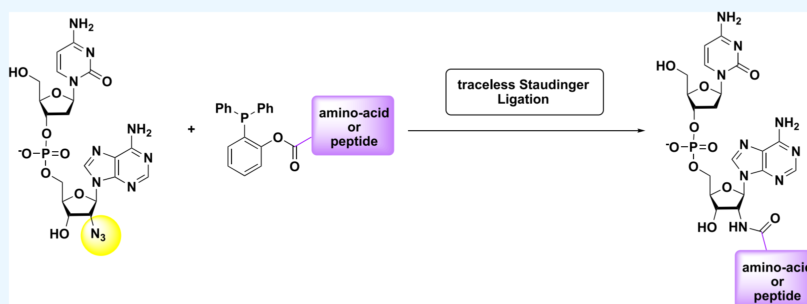

ABSTRACT: Aminoacyl- and peptidyl-tRNA are specific biomolecules involved in many biological processes, from ribosomal protein synthesis to the synthesis of peptidoglycan precursors. Here, we report a post-synthetic approach based on traceless Staudinger ligation for the synthesis of a stable amide bond to access aminoacyl- or peptidyl-di-nucleotide. A series of amino-acid and peptide ester phenyl phosphines were synthetized, and their reactivity was studied on a 2′-N_3 di-nucleotide. The corresponding 2′-amide di-nucleotides were obtained and characterized by LC-HRMS, and mechanistic interpretations of the influence of the amino acid phenyl ester phosphine were proposed. We also demonstrated that enzymatic 5′-OH phosphorylation is compatible with the acylated di-nucleotide, allowing the possibility to access stable aminoacylated-tRNA.

■ INTRODUCTION

Amino acyl- and peptidyl-tRNAs are natural or synthetic[1] biomolecules composed of an RNA part covalently linked to an amino acid or a peptide. The most well-known natural RNAs containing an amino acid residue are the aminoacyl-tRNAs (aa-tRNA), in which the amino acid is connected at a 3′- or 2′-position of the terminal ribose by an ester link (Figure 1). Aa-tRNAs are used in ribosomal protein synthesis but also in non-ribosomal peptide synthesis (NRPS). This NRPS includes the synthesis of peptidoglycan precursors[2] or tetrapyrrole,[3] modification of bacterial membrane lipids,[4] and *N*-terminal labeling of proteins targeted for proteolysis.[5] Aminoacyl-tRNAs also participate in secondary metabolism,[6] including the synthesis of cyclic dipeptides[7] and natural antibiotics.[8]

The exploration of the role of aminoacyl-tRNA in biological processes requires stable molecules. Stable aa-tRNA analogues have been first designed by replacing the terminal ribose by a deoxy-ribose to avoid the isomerization of the amino acid moiety between the 2′- and 3′-hydroxyl of the ribose. The replacement of the ester bond by a stable function such as a triazole ring,[9] an oxadiazole,[10] or an amide bond[11] could also be used to stabilize the link between the amino acid and the RNA (Figure 1). These stable analogues have been efficiently used to obtain crystallographic structures of complexes between aa-tRNA analogues and the enzyme[12] or to decipher

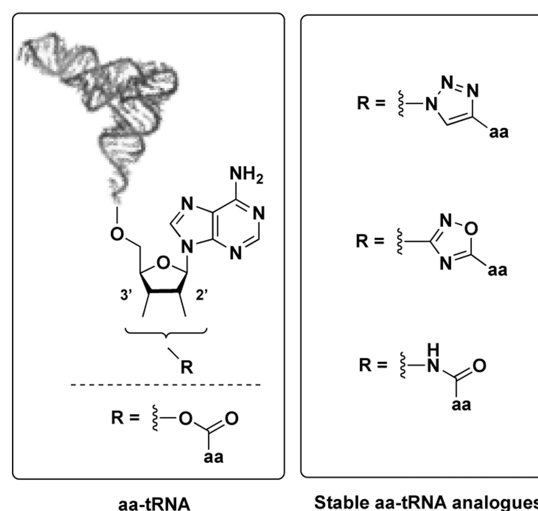

Figure 1. Structure of aa-tRNA and stable analogues.

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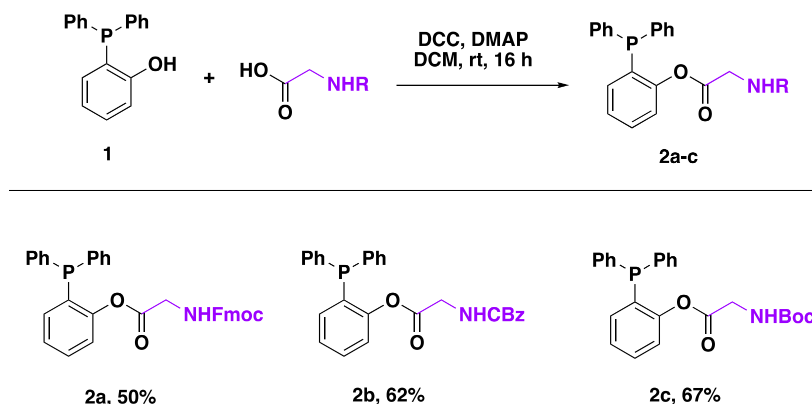
Scheme 1. Synthesis of *N*-Protected Glycine Phosphines



the specificity or regiospecificity of the transfer of the amino acid.[13]

For the synthesis of aminoacyl-RNA analogues containing an amide bond, two approaches have been used: (i) the pre-synthetic approach, which involves the formation of the amide bond on the nucleoside and then its incorporation into the RNA by solid phase synthesis or enzymatic ligation, or (ii) the post-synthetic approach, which involves the post-functionalization of an amino-RNA. The first approach was performed using standard peptide coupling[11,14] or Staudinger−Vilarrasa reaction[15] to link activated amino acids to amino- or azido-deoxyadenosine, respectively. This method requires relatively large amounts of modified nucleotide monomers obtained by multi-step syntheses. The second approach has been described in a single report, in which the direct coupling of an activated amino acid to an 18 nt 3′-amino-RNA has been efficiently used only for formyl-methionine.[11b] The challenge in the post-synthetic strategy is to find a chemoselective reaction, allowing site-specific acylation starting from a non-protected RNA, which contains several other nucleophilic groups. Here we propose to use the chemoselective traceless Staudinger ligation[16] in the presence of amino acids or peptide phosphines for the post-functionalization of an azido-di-nucleotide, which is a known precursor for the synthesis of aa-tRNA.[10,17]

The use of amino acids in the traceless variant of the Staudinger ligation has been first introduced for the synthesis of peptides in the presence of phosphinothiol by the work of Raines.[18] Thanks to this Staudinger peptide ligation (SPL) approach, two peptides have been efficiently ligated from glycine-azido derivatives.[19] The ligation with non-glycinic amino-acid turns out to be more complex since an aza-Wittig by-product could be observed. To overcome this issue, an effort has been made to design a novel phosphinothiol in which the phenyl groups have been substituted by an *O*-methoxy group at the *para* position.[20] Long[21] and cyclic[22] peptides were also synthesized by classical traceless Staudinger ligation using phosphino-ester or, more recently, with 2-(diphenylphosphanyl)phenyl)methane.[23] The group of Bernardi[24] applied such a methodology for the synthesis of amide-substituted glycosyl derivatives, while Guo and co-workers described the preparation of glycan-peptide conjugates.[25] Interestingly, all these examples mainly report the use of phosphines substituted with protected amino acids or peptides, and no study has ever depicted the reactivity of ester phenyl phosphine carrying unprotected amino acids for traceless Staudinger ligation, which could be useful in a post-synthetic strategy.

RESULTS AND DISCUSSION

To explore the traceless Staudinger ligation to access aminoacyl- or peptidyl-RNA analogues, the dCpA-2′-N_3 di-nucleotide **4** containing an azido group at the 2′ position of the terminal adenosine was used. This dinucleotide has already been used in many studies to mimic the terminal CA motif present in all tRNAs, and the 2′-deoxy-cytidine does not impair the functionality of aa-tRNA.[10] The development of post-functionalization reaction conditions went through the preparation of phosphines substituted with an amino acid or a peptide as a second partner for the ligation step. The reactivity of phosphines was investigated, and a mechanistic interpretation was proposed to rationalize the experimental results.

We first started our investigation with the synthesis of NH_2-protected amino acid phenyl ester phosphines with different protecting groups. For this purpose, the commercially available 2-hydroxyphenyl diphenylphosphine **1** was esterified in the presence of protected glycine, DCC, and DMAP in DCM at room temperature for 16 h. Glycine was selected as the model amino acid for our study. This reaction allowed the formation of three glycine phenyl ester phosphines **2a-c** containing a variety of protecting groups including Fmoc, benzyloxycarbonyl (CBz), and Boc in 50, 62, and 67% yields, respectively (Scheme 1).

The phosphines **2a−c** were then submitted to classical deprotection conditions. Under basic conditions (Scheme 2, entry 1), the degradation of the reaction mixture was observed, which led to the oxidation of phosphine and the cleavage of the ester linkage. Compound **2b** was treated with a catalytic amount of Pd/C under hydrogen pressure, and degradation of only the starting material was observed (Scheme 2, entry 2). The synthesis of the NH_2-free glycine phenyl ester phosphine was finally obtained starting from compound **2c** treated with a 15% solution of TFA in DCM at room temperature for 2 h. Compound **2i** was then obtained in 86% yield as a trifluoroacetate salt (Scheme 2, entry 3). This study shows that the best option to protect the amine to synthesize amino acid-phosphine phenol ester is the *tert*-butoxycarbonyl (Boc).

To expand the scope of NH_2-unprotected amino-acid phosphine, a range of five amino-acids and peptides were selected. *N*-Boc-protected phosphines were obtained by coupling an alanine, a valine, and a phenylalanine in the presence of compound **1** using DCC and DMAP according to the route previously described for the synthesis of glycine phenyl ester phosphine (Scheme 1). The desired amino-acid phosphine esters **2d**, **2e**, and **2f** were isolated in 85, 96, and

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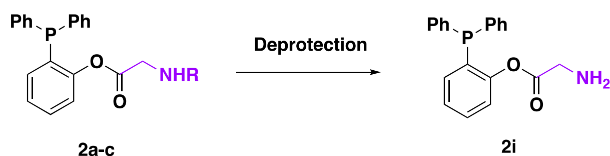
Scheme 2. Optimization of Deprotection Conditions



Entry	Phosphine	Conditions	2i
1	2a	Piperidine 20% DMF, 16h, rt	Degradation
2	2b	H_2, Pd/C THF, 16 h, rt	Degradation
3	2c	TFA 15% DCM, 2 h, rt	86%[a]

[a] compound 2i isolated in a trifluoroacetate salt.

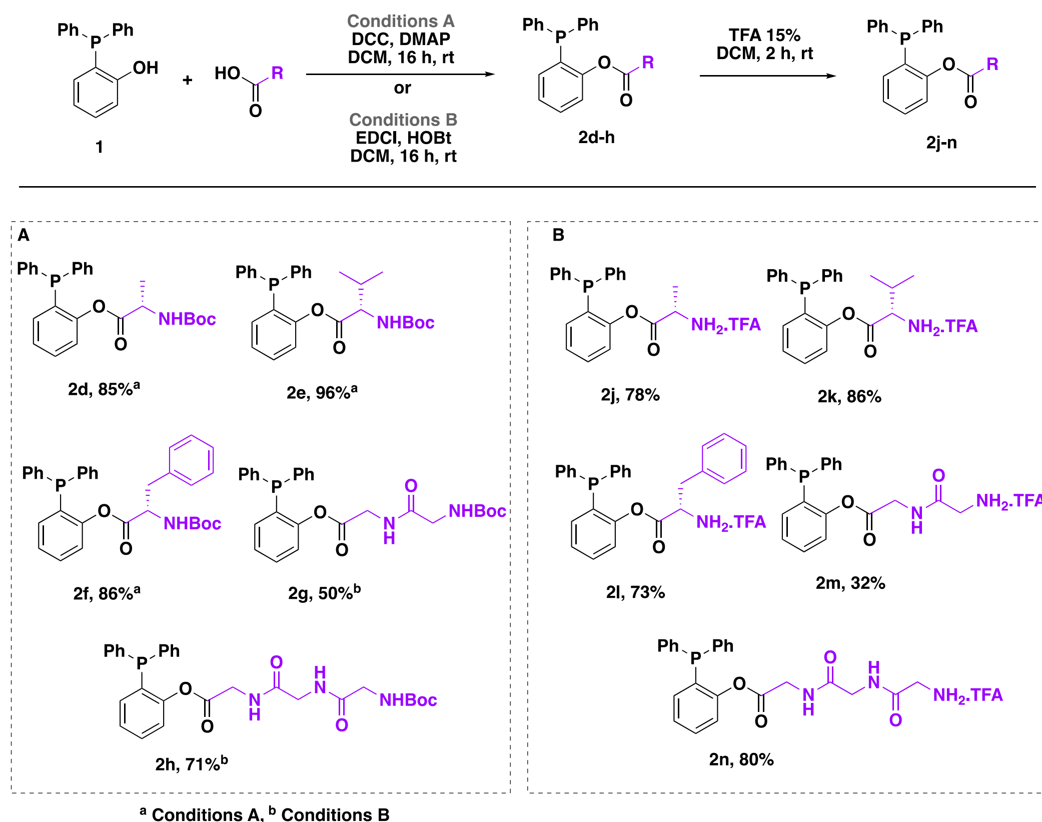
86% yields, respectively (Scheme 3A). Di- and tri-peptides were also engaged in a coupling reaction in the presence of the phenol phosphine **1**, with EDCI and HOBt as coupling reagents (Conditions B, Scheme 3). The corresponding phosphines **2g** and **2h** with a di-glycine and tri-glycine peptide were obtained in 50 and 71% yields, respectively (Scheme 3A). The deprotection conditions using a solution of 15% TFA in DCM were then applied, and five unprotected amino-acid or peptide phenyl ester phosphines **2j**–**n** were isolated in 32 to 80% yields (Scheme 3B).

Among the series of previously synthesized phosphines, we next focused our attention on the reactivity of amino acid and peptide phosphines **2i**, **2j**, **2m,** and **2n** substituted with Gly and Ala groups. The reactions were carried out on 0.83 μmol of di-nucleotide **4** and analyzed by LC-HRMS.

The dinucleotide substrate **4** was first treated in the presence of 10 equivalents of phosphine **2i** and heated for 4 h at 70 °C, followed by the addition of water and 2 h of stirring at 70 °C to get a full conversion. Under these conditions, the formation of the ligated compound **5i** was observed in 12% yield in competition with the reduced derivative **7** (Scheme 4, entry 1). Phosphine **2j** and **2k** lead to the same ratio in favor of the reduced dinucleotide **7** (Scheme 4, entries 2 and 3). When dipeptide **2m** is used, no compound from the traceless Staudinger ligation was detected by LC-HRMS, and the reduction of dinucleotide **4** afforded compound **7**. In addition, a cyclic oxazaphospholane derivative,[26] **6m,** was obtained in 28% yield (Scheme 4, entry 4). The formation of such a cyclic derivative could be explained by the 1,2-*cis* position[27] of the 2′-N_3 function and the 3′-OH hydroxyl of the ribose. In the presence of phosphine **2n** substituted with a glycine tripeptide, di-nucleotide **4** was converted into the expected ligated compound **2n** in 10% yield (Scheme 4, entry 5). The access to the amide function was in competition with the formation of the oxazaphospholane derivative **6n** observed in 47% yield and the reduction of the azide (compound **7**) observed in 37% yield.

Based on the modest yield of traceless Staudinger ligation obtained with unprotected amino acid or peptide phosphines, we next examined the reactivity of *N*-Boc-protected phosphine derivatives. Traceless Staudinger ligation was then applied to di-nucleotide **4** in the presence of protected phosphines **2c**, **2d**,

Scheme 3. Synthesis of Phosphine Phenol Esters Containing (A) *N*-Boc-Protected Amine and (B) Unprotected Amine



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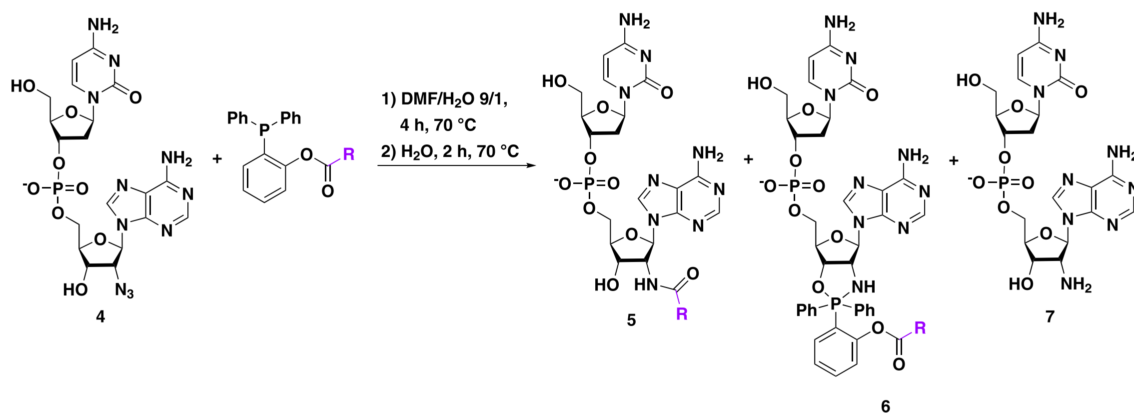
Scheme 4. Substrate Scope



Entry	Phosphine	5[a]	6[a]	7[a]
1	2i (R = CH2NH2.TFA)	5i: 12%	6i: -	88%
2	2j	5j: 18%	6j: -	82%
3	2k	5k: 4%	6k: 8%	88%
4	2m	5m: -	6m: 28%	72%
5	2n	5n: 10%	6n: 47%	37%
6	2c	5c: 29%	6c: 63%	6%
7	2d	5d: 35%	6d 46%	19%
8	2g	5g: 13%	6g: 83%	4%
9	2h	5h: 21%	6h: 48%	31%

[a] Product yield based on LC-HRMS data by relative quantification of dinucleotides.

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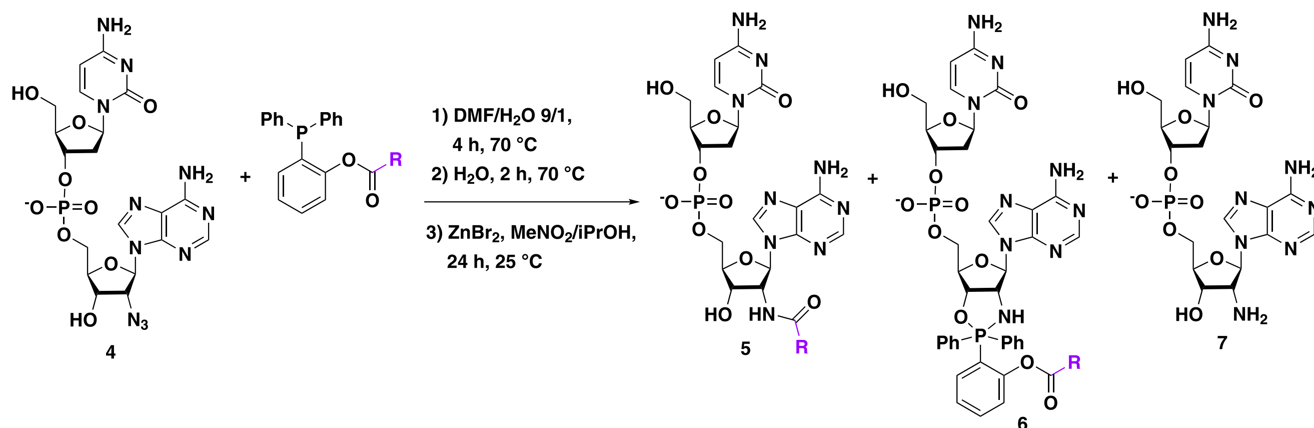
Scheme 5. One-Pot Procedure



Entry	Phosphine	5[a]	6[a]	7[a]
1	2c (Ph, Ph, P; NHBoc)	5i: 13%	6i: 4%	83%
2	2d (Ph, Ph, P; NHBoc)	5j: 30%	6j: -	70%

[a] Product yield based on LC-HRMS data by relative quantification of dinucleotides.

2g, and **2h**. By contrast, the protection of the amino group allowed an increase in the yield of the formation of the amide bond up to 35% for compound **2d**. Although the reduced dinucleotide **7** was observed in the reaction mixture, the yield drastically decreased with protected phosphines (4 to 31% vs 37 to 88%). The formation of the oxazaphospholane derivatives was also detected with different yields (between 46 and 83% yields), depending on the phosphine.

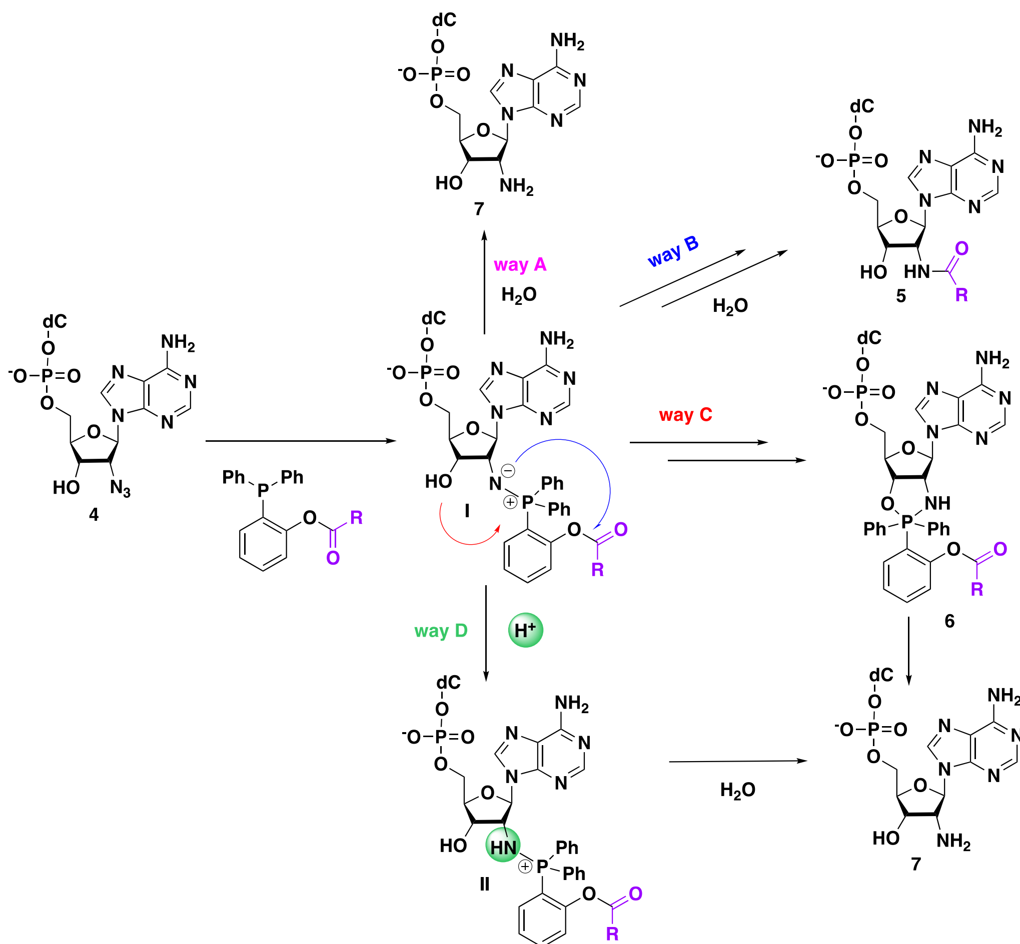
These experimental results indicate that the use of *N*-Boc-protected phenyl ester phosphines impacts the yield of the reaction in favor of the amide derivative. We then reinvestigated our strategy and developed a three-step procedure to ligate a protected amino acid or peptide-phosphine by traceless Staudinger ligation (Scheme 5). After the ligation step of dCpA-2′-N_3 di-nucleotide **4** in the presence of phosphines **2c** and **2d** at 70 °C for 4 h, followed by an additional 2 h of stirring at 70 °C in water, the crude mixture was treated with $ZnBr_2$ salts[28] for 24 h in a mixture of isopropanol and nitromethane. These conditions enabled the formation of the amide derivatives **5i** and **5j** in 13 and 30% yields, starting with phosphine **2c** and **2d.** This three-step procedure allows higher ligation yields than the use of unprotected amino acid or peptide phosphines.

The results observed in this study show that the substituent on the amino group of the amino-acid or peptide linked to the ester phenyl phosphine has a strong impact on the ligation through a traceless Staudinger pathway. When the amino-acid is deprotected, the reduction of azide **4** is favored. In contrast, the yield increased in favor of the desired ligated derivative with *N*-Boc-protected phosphines, but an important amount of cyclic oxazaphospholane could be observed. To rationalize these results, a putative mechanism is proposed in Scheme 6.

After the formation of the iminophosphorane intermediate **I**, Staudinger reduction could lead directly to the formation of the reduced di-nucleotide **7** (way A). In a previous study,[26] we have demonstrated that the reaction occurred in competition with the acyl transfer from the phosphine to the nitrogen on the iminophosphorane through a six-membered ring formed during the transition state (way B) and the formation of the cyclic oxazaphospholane derivative promoted by the vicinal *cis* configuration of the nitrogen of the iminophosphorane and the hydroxyl at the 3′ position (way C). In the presence of *N*-Boc-protected phosphine, the nucleophilic attack of nitrogen on electrophilic carbonyl could be disadvantageous due to the steric hindrance of the Boc group and lead to the formation of compound **6**. With NH_2-free amino acid or peptide phenyl ester phosphines, it offers an additional source of proton (coming from the TFA salt) that could easily protonate the nitrogen at the 3′-position, reported as a strong base,[29] affording intermediate **II**. Then, the hydrolysis with water could provide reduced compound **7**.

The crude mixture obtained after traceless Staudinger ligation with phosphine **2j** was then submitted to enzymatic 5′- phosphorylation using bacteriophage T4 polynucleotide kinase to yield dinucleotide **8** in 24% yield (Scheme 7). This was confirmed by LC-HRMS analyses (see Supporting Information S37-38). An additional enzymatic ligation step, starting from **8**, could provide a series of stable aa-tRNAs since T4 RNA ligase is known to efficiently catalyze the ligation of the 3′-OH terminus of an RNA with a 5′-phosphorylated di-nucleotide.[11a]

Scheme 6. Proposed Mechanism



■ CONCLUSIONS

In conclusion, we have reported the first application of traceless Staudinger ligation to functionalize, in a post-synthetic approach, di-nucleotides with amino-acid or peptide phosphines. We synthesized a series of ester phenyl phosphines containing various amino acids or peptides. Boc was used as the protecting group for the amine since the deprotection step to remove other groups led to the degradation of the phosphine ester. Traceless Staudinger ligation was performed on a 2′-azido di-nucleotide in the presence of the protected and unprotected Gly and Ala phosphines. The use of unprotected phosphine ester provided the direct formation of the amino acid dinucleotide, although with moderate yields (12 and 18% yield). Inversely, the use of the protected amino phosphine ester allows us to obtain a better yield in favor of the amino acid dinucleotide (29 and 35%). We then perform a one-pot three-step procedure in the presence of *N*-BocAla and NBocGly phosphines to form two desired amino acylated di-nucleotides in 13 and 30% yields.

The post-synthetic traceless Staudinger ligation reported here is a versatile method allowing us to obtain a variety of stable amino acyl-tRNAs from a single acylated di-nucleotide. Of note, our synthetic route could also be of interest for studying RNAs containing non-canonical bases modified with amino acids.[30] Indeed, it has recently been suggested that such modifications of RNA could be living molecular fossils, and the group of T. Carell synthesized amino-acyl RNAs to support the idea of a molecular RNA-peptide world.[31]

■ EXPERIMENTAL SECTION

General Information. Reactions were carried out under an argon atmosphere and performed using freshly distilled solvents. THF was dried over sodium/benzophenone; DCM, DMF, and MeOH were dried over calcium hydride. Di-nucleotide **4** was synthesized according to the procedure previously reported by our group.[26] (2-Hydroxyphenyl)diphenylphosphine was purchased from Merck. The progress of the reactions was monitored by thin layer chromatography (TLC). TLC: precoated silica gel thin layer sheets 60 F_{254}(Merck) and detection by charring with 10% H_2SO_4 in ethanol or phosphomolybdic acid in ethanol, followed by heating. Flash chromatography was carried out over silica gel (60 Å, 180−240 mesh) from Merck. Preparative HPLC was performed using a HPLC system with a reverse phase C-18 NUCLEOSIL column (250 mm × 21.2 mm, 5 μm) using a solvent system consisting of A: 50 mM aqueous NH_4OAc pH 4.5 and B: MeCN (linear gradient from 0% B to 63% B in 30 min) at a flow rate of 15 mL/min and UV detection at 254 nm. NMR spectra were recorded on Bruker spectrometers (Avance II 500 and Avance III HD 4000). Chemical shifts (δ) are reported in parts per million (ppm) and referenced to the residual proton or carbon resonance of the solvents: $CDCl_3$ (δ 7.26), D_2O (δ 4.79), DMSO (δ 2.50), CD_3OD (δ 3.31) or D_2O (δ 4.79) for ^1H and $CDCl_3$ (δ 77.16) or $(CD_3)_2SO$ (δ 39.52), or CD_3OD (δ 49.00) for ^{13}C. Signals were assigned using 1D (^1H and ^{13}C) and 2D (HSQC, COSY, and HMBC) experiments. NMR coupling constants (*J*) are reported in

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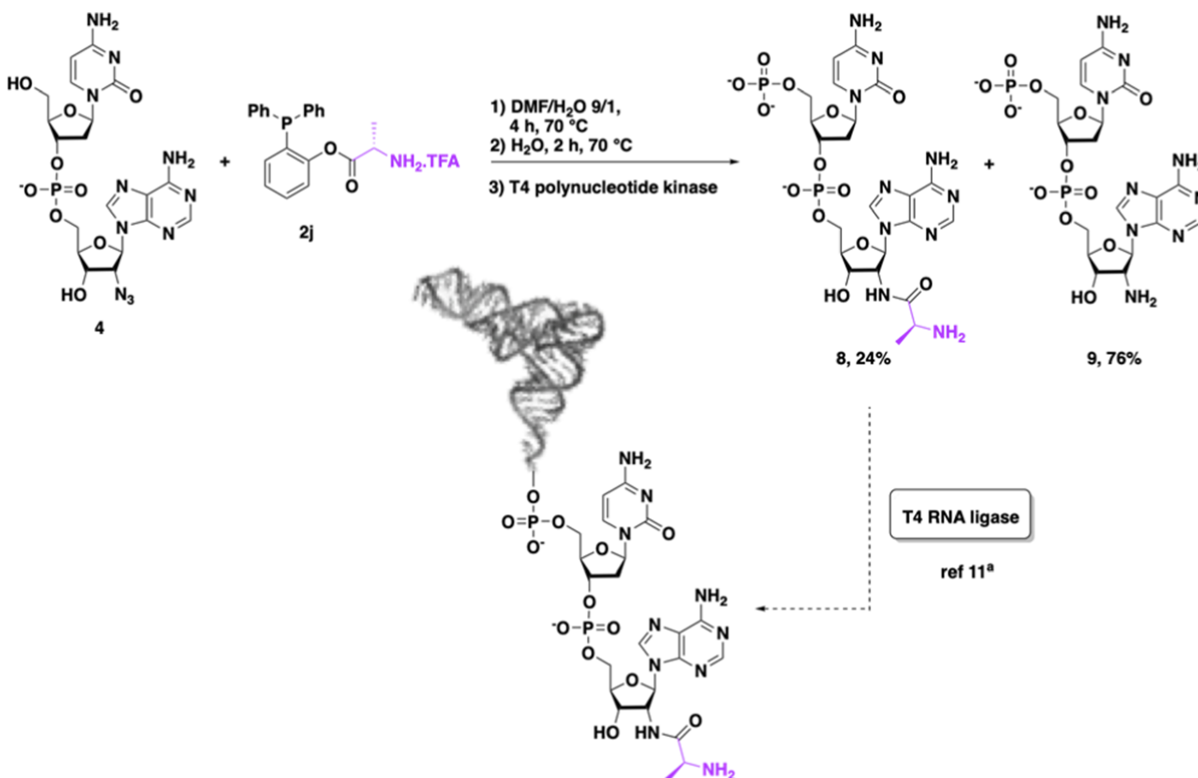
Scheme 7. Access to Stable Aa-tRNA by Enzymatic Phosphorylation and Ligation



Hertz (Hz), and splitting patterns are indicated as follows: s (singlet), bs (broad singlet), d (doublet), t (triplet), q (quartet), dd (doublet of doublet), and m (multiplet).

LC/HRMS Conditions: Conditions A. HPLC analysis was performed on a Hypersil GOLD aQ (ThermoFisher Scientific) column (100 mm × 2.1 mm, 1.9 μm) using a solvent system consisting of A: H_2O with 0.02% AcOH and B: MeCN with 0.02% AcOH (linear gradient from 0 to 100% B in 30 min) at a flow rate of 0.2 mL/min at 30 °C and UV detection at 254 nm. Fractions detected using low-resolution mass spectra (HRMS) were obtained on a LC QTOF mass spectrometer. **Conditions B:** LC-HRMS analyses were performed on a Q Exactive mass spectrometer (Thermo Fisher Scientific), equipped with an electrospray ionization source (H-ESI II probe) coupled with an Accela 1250 Pump (Thermo Fisher Scientific). Samples were injected onto a Thermo Fisher Hypersil GOLD aQ chromatography column (100 mm × 2.1 mm, 1.9 μm particle size). The flow rate was set at 0.3 mL/min, and the mobile phase consisted of water containing 0.02% acetic acid (solvent A) and acetonitrile containing 0.02% acetic acid (solvent B). The gradient program was as follows: 0 to 5 min, 2% B; 5 to 20 min, up to 70% B; 20 to 30 min, came back to 98% B and 2 min of equilibration. The column temperature was maintained at 30 °C, and the temperature of the autosampler was set at 4 °C. MS analyses were performed in a full scan negative ion mode with a scan range from 200 to 1500 *m/z*.

General Procedure 1 for the Preparation of Phosphine 2a-f: Conditions A. To a solution of (2-hydroxyphenyl)diphenylphosphine (1 equiv) in DCM were successively added DCC (3 equiv), DMAP (4 equiv), and a *N*-protected amino acid (3 equiv). The mixture was stirred overnight at room temperature, then filtered through a pad of Celite, and concentrated under reduced pressure. The crude product was purified by flash chromatography using cyclohexane/EtOAc as an eluent to afford the desired phosphine.

General Procedure 2 for the Preparation of Phosphine 2g-h: Conditions B. To a solution of *N*-Boc di- or tri-peptide (1 equiv) in DCM were successively added EDCI (2 equiv) and HOBt (2 equiv). The mixture was stirred for 30 min at room temperature, and (2-hydroxyphenyl) diphenylphosphine (2 equiv) was added at 0 °C. The reaction mixture was then stirred at room temperature for 16 h. DCM and brine were added, and the organic phase was washed three times with brine. The organic layer was dried over $MgSO_4$, filtered, and concentrated. The residue was purified by silica gel chromatography using cyclohexane/EtOAc as the eluent to afford the desired phosphine.

*Compound (**2a**).* Following general procedure 1 for the preparation of amino acid-phosphine, compound **2a** was obtained (150 mg, 50%) as a white foam after purification by flash chromatography using cyclohexane/EtOAc (7/3) as the eluent, starting from (2-hydroxyphenyl) diphenylphosphine (150 mg, 0.54 mmol) and *N*-Fmoc-Gly (480 mg, 1.62 mmol) in DCM (5 mL). ^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.70 (d, J = 7.5 Hz, 2 H, H^{Ar}), 7.53 (d, J = 7.3 Hz, 2 H, H^{Ar}), 7.34 (d, J = 7.3 Hz, 2 H, H^{Ar}), 7.28−7.24 (m, 13 H, H^{Ar}), 7.11−7.08 (m, 2 H, H^{Ar}), 6.83−6.80 (m, 1 H, H_{Ar}), 4.99 (br s, 1 H, NH), 4.34 (d, J = 7.0 Hz, 2 H, CH_2^{Fmoc}), 4.18−4.15 (m, 1 H, CH^{Fmoc}), 3.85−3.84 (m, 2 H, CH_2^{Gly}). ^{13}C NMR (126 MHz, $CDCl_3$): δ = 168.1 (C=O), 156.1 (C=O), 152.4, 143.9, 141.4, 135.4, 134.2, 134.0, 130.4, 130.2, 129.3, 128.8, 127.8, 127.2, 126.7, 125.2, 122.5, 120.1, 67.3 (CH_2^{Fmoc}), 47.3 (CH^{Fmoc}), 42.8 (CH_2^{Gly}). ^{31}P NMR (202 MHz, $CDCl_3$): δ = −15.7. HRMS (ESI) *m/z*: $[M + H]^+$ calcd for $C_{35}H_{29}NO_4P$ 558.18342; found, 558.18175.

*Compound (**2b**).* Following general procedure 1 for the preparation of amino acid-phosphine, compound **2b** was

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obtained (157 mg, 62%) as a white foam, after purification by flash chromatography using cyclohexane/EtOAc (8/2) as the eluent, starting from (2-hydroxyphenyl) diphenylphosphine (150 mg, 0.54 mmol) and *N*-Cbz-Gly (338 mg, 1.62 mmol) in DCM (10 mL). ^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.28−7.23 (m, 16 H, 5 H^{ArCbz}, 12 H^{ArPhos}), 7.08−7.05 (m, 2 H, H^{ArPhos}), 6.78−6.76 (m, 1 H, H^{ArPhos}), 5.02 (s, 2 H, CH_2^{Cbz}), 3.80−3.79 (m, 2 H, CH_2^{Gly}). ^{13}C NMR (126 MHz, $CDCl_3$): δ = 168.1 (C═O), 156.2 (C═O), 152.3, 136.3, 135.4, 135.3, 134.1 (2C), 133.9 (2C), 130.5, 130.3, 130.2, 129.3 (2C), 128.8 (2C), 128.7 (2C), 128.6 (2C), 128.5, 128.3, 128.2, 126.7, 122.5, 67.21 (CH_2^{Cbz}), 42.71 (CH_2^{Gly}). ^{31}P NMR (202 MHz, $CDCl_3$): δ = −15.8. HRMS (ESI) *m*/*z*: $[M + H]^+$ calcd for $C_{28}H_{25}NO_4P$ 470.15212; found, 470.15210.

Compound (2c). Following general procedure 1 for the preparation of amino acid-phosphine, compound **2c** was obtained (262 mg, 67%) as a white foam, after purification by flash chromatography using cyclohexane/EtOAc (8/2) as the eluent, starting from (2-hydroxyphenyl) diphenylphosphine (250 mg, 0.89 mmol) and *N*-Boc-Gly (472 mg, 2.67 mmol) in DCM (10 mL). ^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.40−7.33 (m, 11 H, H^{Ar}), 7.20−7.16 (m, 2 H, H^{Ar}), 6.90−6.88 (m, 1 H, H^{Ar}), 4.70 (br s, 1 H, NH), 3.84 (d, *J* = 5.0 Hz, 2 H, CH_2^{Gly}), 1.49 (s, 9 H, tBu^{Boc}). ^{13}C NMR (126 MHz, $CDCl_3$): δ = 168.5, 155.6, 152.3, 135.5, 134.1, 134.0, 133.7, 133.5, 130.5, 130.4, 130.2, 129.3, 129.2, 128.8, 128.7, 126.6, 122.6, 80.1 (Cq^{Boc}), 42.4 (CH_2^{Gly}), 28.4 (tBu^{Boc}). ^{31}P NMR (202 MHz, $CDCl_3$): δ = −15.7. HRMS (ESI) *m*/*z*: $[M + H]^+$ calcd for $C_{25}H_{27}NO_4P$ 436.16777; found, 436.16634.

Compound (2d). Following general procedure 1 for the preparation of amino acid-phosphine, compound **2d** was obtained (276 mg, 85%) as a white foam, after purification by flash chromatography using cyclohexane/EtOAc (8/2) as the eluent, starting from (2-hydroxyphenyl) diphenylphosphine (200 mg, 0.7 mmol) and *N*-Boc-Ala (407 mg,2.1 mmol) in DCM (10 mL). ^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.28−7.17 (m, 11 H, H^{Ar}), 7.09−7.06 (m, 1 H, H^{Ar}), 7.02 (t, *J* = 7.5 Hz, 1 H, H^{Ar}), 6.71−6.69 (m, 1 H, H^{Ar}), 4.82 (br s, 1 H, NH), 4.26 (br s, 1 H, Hα), 1.35 (s, 9 H, tBu^{Boc}), 1.16 (d, *J* = 5 Hz, 3 H, CH_3^{Ala}). ^{13}C NMR (126 MHz, $CDCl_3$): δ = 171.2, 152.6, 135.4, 134.0, 133.8, 133.5, 133.3, 131.9, 132.5, 130.0, 129.9, 129.1, 128.9, 128.6, 126.4, 122.2, 121.0, 116.1, 79.8 (Cq^{Boc}), 49.5 (CHα), 28.3 (tBu^{Boc}), 18.1 (CH_3^{Ala}). ^{31}P NMR (202 MHz, $CDCl_3$): δ = −16.8. HRMS (ESI) *m*/*z*: $[M]^+$ calcd for $C_{26}H_{28}NO_4P$ 449.1555; found, 449.1744.

Compound (2e). Following general procedure 1 for the preparation of amino acid-phosphine, compound **2e** was obtained (329 mg, 96%) as a white foam, after purification by flash chromatography using cyclohexane/EtOAc (9/1) as the eluent, starting from (2-hydroxyphenyl) diphenylphosphine (200 mg, 0.7 mmol) and *N*-Boc-Val (390 mg, 2.1 mmol) in DCM (10 mL). ^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.39−7.32 (m, 7 H, H^{Ar}), 7.31−7.37 (m, 4 H, H^{Ar}), 7.19−7.17 (m, 1 H, H^{Ar}), 7.14 (t, *J* = 10 Hz, 1 H, H^{Ar}), 6.83−6.81 (m, 1 H, H^{Ar}), 4.88 (d, *J* = 10 Hz, 1 H, NH), 4.29−4.27 (m, 1 H, Hα), 4.18−4.16 (m, 1 H, Hβ), 1.46 (s, 9 H, tBu^{Boc}), 0.94 (d, *J* = 5 Hz, 3 H, CH_3^{Val}), 0.82 (d, *J* = 5 Hz, 3 H, CH_3^{Val}). ^{13}C NMR (126 MHz, $CDCl_3$): δ = 170.4, 155.8, 153.1, 152.9, 135.9, 135.8, 135.7, 134.2, 134.1, 134.0, 130.3, 130.2, 129.3, 129.2, 128.9, 128.8, 126.5, 122.3, 79.8 (Cq^{Boc}), 58.8 (Cα), 31.1 (Cβ), 28.5 (tBu^{Boc}), 19.4 (CH_3^{Val}), 17.3 (CH_3^{Val}). ^{31}P NMR (202 MHz, $CDCl_3$): δ = −17.2. HRMS (ESI) *m*/*z*: $[M]^+$ calcd for $C_{28}H_{32}NO_4P$ 477.2069; found, 477.2057.

Compound (2f). Following general procedure 1 for the preparation of amino acid-phosphine, compound **2f** was obtained (324 mg, 86%) as a white foam, after purification by flash chromatography using cyclohexane/EtOAc (9/1) as the eluent, starting from (2-hydroxyphenyl) diphenylphosphine (200 mg, 0.7 mmol) and *N*-Boc-Phe (551 mg, 2.1 mmol) in DCM (10 mL). ^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.41−7.31 (m, 11 H, H^{Ar}), 7.30−7.26 (m, 2 H, H^{Ar}), 7.24−7.20 (m, 1 H, H^{Ar}), 7.15−7.12 (m, 4 H, H^{Ar}), 6.84−6.81 (m, 1 H, H^{Ar}), 4.78 (br s, 1 H, NH), 4.52 (br s, 1 H, Hα), 3.14−3.10 (m, 1 H, $CH_2\beta$), 2.80−2.76 (m, 1 H, $CH_2\beta$), 1.39 (s, 9 H, tBu^{Boc}). ^{13}C NMR (126 MHz, $CDCl_3$): δ = 169.8, 155.1, 152.7, 136.6, 135.5, 134.2, 134.0, 133.9, 133.6, 133.4, 130.1, 129.6, 129.2, 129.1, 128.8, 128.6, 127.0, 126.5, 122.2, 121.7, 116.2, 80.0 (Cq^{Boc}), 54.7 (CHα), 31.9 (CH_2^{Phe}), 28.4 (tBu^{Boc}). ^{31}P NMR (202 MHz, $CDCl_3$): δ = −16.6. HRMS (ESI) *m*/*z*: $[M]^+$ calcd for $C_{32}H_{32}NO_4P$ 525.2069; found, 525.2051.

Compound (2g). Following general procedure 2 for the preparation of amino acid-phosphine, compound **2g** was obtained (260 mg, 50%) as a white foam, after purification by flash chromatography using cyclohexane/EtOAc (6/4) as the eluent, starting from (2-hydroxyphenyl) diphenylphosphine (300 mg, 1.0 mmol), EDCI (412 mg, 2.0 mmol), HOBt (290 mg, 2.0 mmol), and *N*-Boc-GlyGly (505 mg, 2.0 mmol) in DCM (10 mL). ^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.36−7.11 (m, 13 H, H^{Ar}), 6.80−6.74 (m, 1 H, H^{Ar}), 3.91−3.87 (m, 2 H, CH_2), 3.71−3.67 (m, 2 H, CH_2), 1.40 (s, 9 H, tBu^{Boc}). ^{13}C NMR (126 MHz, $CDCl_3$): δ = 171.5 (C═O), 1697.9 (C═O), 159.2, 156.9 (C═O), 152.8, 136.7, 135.5, 134.4, 133.6, 133.5, 133.4, 133.3, 129.9, 129.8, 128.8, 128.3, 128.2, 127.9, 126.1, 122.2, 119.4, 114.2, 79.3 (Cq^{Boc}), 40.4 (CH_2), 40.3 (CH_2), 27.2 (2 tBu^{Boc}), 24.6(tBu^{Boc}). ^{31}P NMR (202 MHz, $CDCl_3$): δ = −19.4 HRMS (ESI) *m*/*z*: $[M + H]^+$ calcd for $C_{27}H_{30}N_2O_5P$ 493.18923; found, 493.18743.

Compound (2h). Following general procedure 2 for the preparation of amino acid-phosphine, compound **2h** was obtained (140 mg, 71%) as a white foam, after purification by flash chromatography using cyclohexane/EtOAc (3/7) as the eluent, starting from (2-hydroxyphenyl) diphenylphosphine (100 mg, 0.36 mmol), EDCI (138 mg, 0.72 mmol), HOBt (97 mg, 0.72 mmol), and *N*-Boc-GlyGlyGly (208 mg, 0.72 mmol) in DCM (10 mL). ^{1}H NMR (500 MHz, MeOD): δ = 7.36−7.27 (m, 7 H, H^{Ar}), 7.24−7.21 (m, 4 H, H^{Ar}), 7.14−7.11 (m, 2 H, H^{Ar}), 6.81−6.79 (m, 1 H, H^{Ar}), 3.89 (s, 2 H, CH_2^{Gly}), 3.86 (s, 2 H, CH_2^{Gly}), 3.71 (s, 2 H, CH_2^{Gly}), 1.41 (s, 9 H, tBu^{Boc}). ^{13}C NMR (126 MHz, MeOD): δ = 173.0, 122.0, 169.3, 158.3, 155.6, 138.2, 136.9, 136.8, 134.9, 134.7, 134.5, 131.7, 131.6, 131.4, 131.1, 130.2, 129.8, 129.7, 129.6, 129.4, 129.3, 127.5, 123.6, 120.8, 115.9, 80.9 (Cq^{Boc}), 43.1 (CH_2^{Gly}), 41.8 (CH_2^{Gly}), 41.7 (CH_2^{Gly}), 28.7 (tBu^{Boc}). ^{31}P NMR (202 MHz, MeOD): δ = −17.2. HRMS (ESI) *m*/*z*: $[M + H]^+$ calcd for $C_{29}H_{33}N_3O_6P$ 550.21070; found, 550.20908.

General Procedure 3 for the Deprotection of the Boc-Protecting Group. *N*-Boc amino acid/peptide-phosphine (1 equiv) was suspended in a 2 M TFA solution (11 equiv) in DCM, and the reaction was stirred for 2h at room temperature. The reaction mixture was concentrated under reduced pressure, and the residue was purified by flash chromatography (DCM/MeOH 9/1) to yield the corresponding TFA salt.

Compound (2i). Following general procedure 3 for the deprotection of *N*-Boc amino acid-phosphine, compound **2i** was obtained (49 mg, 86%) as a white foam, after the removal

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of TFA by co-evaporation (3 × toluene, 3 × DCM), starting from compound **2c** (55 mg, 0.12 mmol) and TFA (0.69 mL, 1.38 mmol). ^{1}H NMR (500 MHz, MeOD): δ = 7.49−7.46 (m, 1 H, H^{Ar}), 7.39−7.38 (m, 6 H, H^{Ar}), 7.30−7.24 (m, 6 H, H^{Ar}), 6.91−6.89 (m, 1 H, H^{Ar}), 3.82 (s, 2 H, CH$_2$^{Gly}). ^{13}C NMR (126 MHz, MeOD): δ = 167.2, 153.7, 136.7, 135.3, 134.9, 134.7, 132.8, 131.6, 130.4, 130.1, 129.8, 129.6, 128.2, 123.3, 40.9 (CH$_2$^{Gly}). ^{31}P NMR (202 MHz, MeOD): δ = −17.6.^{19}F NMR (471 MHz, MeOD): δ = −76.9. HRMS (ESI) *m*/*z*: [M + H]$^+$ calcd for $C_{20}H_{19}NO_2P$ 336.11479; found, 336.11480.

Compound (2j). Following general procedure 3 for the deprotection of *N*-Boc amino acid-phosphine, compound **2j** was obtained (212 mg, 78%) as a white foam, after purification by flash chromatography using DCM/MeOH (9/1) as the eluent, starting from compound **2d** (262 mg, 0.58 mmol) and TFA (3.2 mL, 6.38 mmol). ^{1}H NMR (500 MHz, CDCl$_3$): δ = 7.27−7.20 (m, 7 H, H^{Ar}), 7.18−7.14 (m, 4 H, H^{Ar}), 7.06−7.01 (m, 2 H, H^{Ar}), 6.71−6.69 (m, 1 H, H^{Ar}), 3.89−3.82 (m, 1 H, Hα), 1.26 (d, *J* = 5 Hz, 3 H, CH$_3$^{Ala}). ^{13}C NMR (126 MHz, CDCl$_3$): δ = 168.5, 152.1, 152.0, 135.1, 135.0, 134.9, 134.0, 133.9, 130.4, 130.0, 129.9, 129.4, 128.9, 128.8, 122.1, 49.3 (Cα), 15.3 (CH$_3$^{Ala}). ^{31}P NMR (202 MHz, CDCl$_3$): δ = −17.1.^{19}F NMR (471 MHz, CDCl$_3$): δ = −75.8. HRMS (ESI) *m*/*z*: [M]$^+$ calcd for $C_{21}H_{20}NO_2P$ 349.1231; found 349.1230.

Compound (2k). Following general procedure 3 for the deprotection of *N*-Boc amino acid-phosphine, compound **2k** was obtained (239 mg, 86%) as a white foam, after purification by flash chromatography using DCM/MeOH (96/4) as the eluent, starting from compound **2e** (271 mg, 0.56 mmol) and TFA (3.1 mL, 6.16 mmol). ^{1}H NMR (500 MHz, CDCl$_3$): δ = 7.39−7.32 (m, 7 H, H^{Ar}), 7.29−7.22 (m, 5 H, H^{Ar}), 6.84−6.82 (m, 1 H, H^{Ar}), 3.86 (br s, 1 H, Hα),2.30−2.27 (m, 1 H, Hβ), 1.00 (d, *J* = 5 Hz, 3 H, CH$_3$^{Val}), 0.92 (d, *J* = 5 Hz, 3 H, CH$_3$^{Val}). ^{13}C NMR (126 MHz, CDCl$_3$): δ = 167.8, 152.5, 152.4, 135.4, 135.3, 134.2, 134.1, 134.0, 133.9, 133.8, 130.4, 129.7, 129.3, 128.9, 127.0, 122.1, 58.7 (Cα), 29.4 (Cβ), 16.9 (CH$_3$^{Val}). ^{31}P NMR (202 MHz, CDCl$_3$): δ = −17.6.^{19}F NMR (471 MHz, CDCl$_3$): δ = −75.6. HRMS (ESI) *m*/*z*: [M]$^+$ calcd for $C_{23}H_{24}NO_2P$ 377.1545; found, 377.1544.

Compound (2l). Following general procedure 3 for the deprotection of *N*-Boc amino acid-phosphine, compound **2l** was obtained (170 mg, 73%) as a white foam, after purification by flash chromatography using DCM/MeOH (96/4) as the eluent, starting from compound **2f** (227 mg, 0.43 mmol) and TFA (2.37 mL, 4.73 mmol). ^{1}H NMR (500 MHz, CDCl$_3$): δ = 7.41−7.32 (m, 11 H, H^{Ar}), 7.31−7.24 (m, 3 H, H^{Ar}), 7.20−7.13 (m, 4 H, H^{Ar}), 6.85−6.83 (m, 1 H, H^{Ar}), 3.73−3.71 (m, 1 H, Hα), 3.11 (dd, *J* = 5, 10 Hz, 1 H, Hβ), 2.83−2.78 (m, 1 H, Hβ). ^{13}C NMR (126 MHz, CDCl$_3$): δ = 171.0, 159.2, 152.4, 136.0, 135.3, 134.6, 134.4, 134.0, 133.9, 133.6, 133.5, 131.5, 130.2, 129.6, 129.3, 129.0, 128.9, 128.8, 128.7, 127.3, 126.6, 122.2, 121.3, 121.0, 115.6, 55.4 (Cα), 38.6 (Cβ). ^{31}P NMR (202 MHz, CDCl$_3$): δ = −17.1.^{19}F NMR (471 MHz, CDCl$_3$): δ = −75.5. HRMS (ESI) *m*/*z*: [M]$^+$ calcd for $C_{27}H_{24}NO_2P$ 425.1544; found, 425.1551.

Compound (2m). Following general procedure 3 for the deprotection of *N*-Boc peptide-phosphine, compound **2m** was obtained (20 mg, 32%) as a white foam, after purification by HPLC starting from compound **2g** (60 mg, 0.12 mmol) and TFA (0.7 mL, 1.32 mmol). ^{1}H NMR (500 MHz, CDCl$_3$): δ = 7.27−7.15 (m, 11 H, H^{Ar}), 7.01−6.96 (m, 2 H, H^{Ar}), 6.74−6.71 (m, 1 H, H^{Ar}), 3.74 (m, 2 H, CH$_2$), 3.6 (s, 2 H, CH$_2$). ^{13}C NMR (126 MHz, CDCl$_3$): δ = 168.2 (C═O), 167.0 (C═O), 152.4, 135.4, 135.3, 134.1, 133.9, 132.2, 132.1, 130.3, 130.4, 129.3 (2C), 128.9 (2C), 128.8 (2C), 126.7, 122.6, 41.2 (CH$_2$^{Gly}), 41.6 (CH$_2$^{Gly}). ^{31}P NMR (202 MHz, CDCl$_3$): δ = −16.2. HRMS (ESI) *m*/*z*: [M + H]$^+$ calcd for $C_{22}H_{22}N_2O_3P$ 393.13626; found, 393.13669.

Compound (2n). Following general procedure 3 for the deprotection of *N*-Boc peptide-phosphine, compound **2n** was obtained (45 mg, 80%) as a white foam, after purification by HPLC, starting from compound **2h** (60 mg, 0.1 mmol) and TFA (0.55 mL, 1.1 mmol). ^{1}H NMR (500 MHz, MeOD): δ = 7.44−7.16 (m, 13 H, H^{Ar}), 6.89−6.84 (m, 1 H, H^{Ar}), 3.96 (d, *J* = 5.25, 2 H; CH$_2$), 3.74 (s, 2 H, CH$_2$), 3.26 (s, 2H, CH$_2$). ^{13}C NMR (126 MHz, MeOD): δ = 171.6 (C═O), 169.4 (C═O), 168 (C═O), 154, 136.9, 136.8, 135.0 (2C), 134.8 (2C), 131.8, 131.7, 131.3, 130.3 (2C), 129.8 (2C), 129.7 (2C), 127.6, 129.6, 42.9 (CH$_2$^{Gly}), 41.9 (CH$_2$^{Gly}), 41.5 (CH$_2$^{Gly}). ^{31}P NMR (202 MHz, MeOD): δ = −17.1. HRMS (ESI) *m*/*z*: [M + H]$^+$ calcd for $C_{24}H_{25}N_3O_4P$ 450.15772; found, 450.15811.

General Procedure 4 for Traceless Staudinger Ligation with Di-nucleotide 4. To a solution of di-nucleotide **4** (0.83 μmol,1 equiv) in an Eppendorf tube in DMF/H$_2$O (9/1, 1 mL) was added phosphine (10 equiv), and the mixture was stirred for 4 h at 70 °C in a thermo-shaker plate. Then, an excess of water (3mL) was added, and the mixture was stirred for 2 h at 70 °C. After lyophilization, the residue was analyzed by LC/HRMS.

General Procedure 5 for Traceless Staudinger Ligation with Di-nucleotide 4 with *N*-Boc-Protected Amino Acid Phosphine. To a solution of di-nucleotide **4** (0.5 mg, 0.83 μmol) in an Eppendorf tube in DMF/H$_2$O (9/1, 1 mL) was added phosphine **2c** (3,7 mg, 8.3 μmol) or **2d** (3,8 mg, 8.3 μmol), and the mixture was stirred for 4 h at 70 °C in a thermo-shaker plate. Then, an excess of water (3mL) was added, and the mixture was stirred for 2 h at 70 °C. After lyophilization, the residue was dissolved in a 5 M solution of ZnBr$_2$ in a 1/1 (v/v) mixture of iPrOH/MeNO$_2$ (69 μL, 344 μmol), and the reaction mixture was stirred at room temperature for 24 h. Water was added, and the mixture was lyophilized and analyzed by UPLC-HRMS.

General Procedure 6 for Traceless Staudinger Ligation and Enzymatic Phosphorylation. To a solution of dinucleotide **4** (1 mg, 1.67 μmol) in DMF/H$_2$O (9/1, 1 mL) in an Eppendorf tube was added phosphine (7 mg, 16.7 μmol), and the mixture was stirred for 4 h at 70 °C in a thermo-shaker plate (Grant Bio). Then, an excess of water (3 mL) was added, and the mixture was stirred for 2 h at 70 °C. After lyophilization, the residue was resuspended in 770 μL of water. The phosphorylation reaction was then carried out in a 1 mL reaction containing 70 mM Tris−HCl pH7.6, 10 mM MgCl$_2$, 5 mM DTT, 5 mM ATP, and 200 μL of T4 polynucleotide kinase (New England Biolabs ref #M0201L) for 3 h at 37 °C. T4 PNK was then inactivated for 20 min at 65 °C. The crude mixture was then analyzed by LC-HRMS.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.2c06135.

> LC/HRMS analysis for compound **5c−d**, **5g−k**, **5m−n**, **6c−d**, **6g−h**, **6m−n**, **7**, **8**, and **9**NMR spectra for compounds **2a−n** FAIR data, including the primary NMR FID files (PDF)

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Notes

The authors declare no competing financial interest.

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