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# Phosphoramidate-peptide synthesis by solution- and solid-phase Staudinger-phosphite reactions‡

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**The chemoselective incorporation of phosphoramidate moieties into peptides by a Staudinger-phosphite reaction of azides can be performed in many solvents, including water. In this report, we present two strategies for an efficient synthesis of phosphoramidate-containing peptides, in which the Staudinger-phosphite reaction is performed either on the solid support or in solution with aryl azido-containing peptides. The corresponding Staudinger reactions proceed in high conversion rates and deliver phosphoramidate peptides, in which the modification site is located in the middle of the peptide sequence. Copyright © 2010 European Peptide Society and John Wiley & Sons, Ltd.**

*Supporting information may be found in the online version of this article*

**Keywords:** Staudinger-phosphite reaction; solid supported reaction; phosphoramidate; aryl azides; chemoselective reaction

Organic azides are very popular functionalities [1], especially among biological chemists since the first reports addressing them in chemoselective transformations, for instance, in the Staudinger ligation and 1,3-dipolar cycloadditions, became available [2–9]. This reporter moiety has been incorporated in a number of biologically relevant macromolecules [10–12], including peptides and proteins [13,14], and addressed in chemoselective reactions that allowed a site-specific incorporation of many biophysical probes or functional modules, including fluorophores [15], biotin [16], PEG [17], or lipids [18]. Additionally, aryl azides can undergo a light-induced transformation to nitrenes, which are first converted into benzazirines or azacycloheptatetraenes before nucleophiles add spontaneously to produce stable covalent linkages [19].

Recently, our group has employed a chemoselective Staudinger-phosphite reaction for the transformation of azides into phosphoramidates, in which the phosphoramidate moiety mimics a naturally phosphorylated tyrosine in a protein [20]. In those studies, a Staudinger reaction of P(III)-compounds was successfully performed on *p*-azido-L-phenylalanine (Pap), containing peptides in organic solvents, as well as in buffers at physiological pH. All peptide substrates that participated in these transformations were prepared utilizing a standard Fmoc-based solid-phase peptide synthesis (SPPS) and always contained Pap at the *N*-terminus.

In subsequent investigations, we intended to synthesize aryl azidopeptides to allow for a later incorporation of phosphoramidates by a Staudinger-phosphite reaction regardless of the peptide sequence. In contrast to the synthesis of alkyl azidopeptides, which can be prepared by standard SPPS protocols in high yields [21,22], we encountered problems during the SPPS of aryl azidopeptides **1**. Specifically, we observed that additional peptide coupling and deprotection cycles after installation of Pap led to the degradation of the aryl azide and that the level of its decomposition was approximately proportional to the number of deprotections/couplings performed (Scheme 1A). In this regard, a Boc-based SPPS that minimizes the exposure to nucleophiles appears to be beneficial [23]. However, we additionally observed instability of Pap under acidic conditions during the cleavage of the peptide from the solid support upon prolonged treatment with 95% trifluoroacetic acid (TFA). Although in this case the degradation of Pap proceeds to a limited extent, a repeated *N*-Boc-deprotection step performed under acidic conditions could result in a major damage to the azide, thus questioning the suitability of Boc-SPPS for the preparation of Pap containing peptides. In an alternative strategy, Pap can be conveniently installed into peptides by diazotization of *p*-aminophenylalanine (Apa) in solution after cleavage from the solid support, which has been demonstrated to proceed in the presence of various functional groups. The efficiency of the transformation of Apa into Pap seems to be sequence dependent, yet the specific amino acids responsible for decreasing the conversion could not be identified based on the available literature reports [24–33]. Furthermore, when Met is present in the peptide sequence, the thioether is expected to undergo oxidation during the diazotization reaction in water [24].

In order to provide general synthetic protocols for the preparation of phosphoramidate peptides, we have now developed two synthetic routes, which involve a Staudinger-phosphite reaction of aryl azidopeptides either in solution or on the solid

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support (Scheme 1B). Whereas the Staudinger-phosphite reaction is performed in the latter strategy during SPPS, the solution-phase protocol employs an azide on the full length peptide as a substrate. It is important to note that each method would have certain advantages. A solution-phase strategy can be considered as convergent with respect to the formation of the phosphoramidate moiety. This represents an ideal strategy when sensitive modules (to a base or to an acid) are introduced by the Staudinger-phosphite reaction, since these functionalities could suffer from conventional Fmoc-deprotection or cleavage conditions. A solid-phase strategy applied for more robust phosphoramidates would allow the removal of the remaining phosphite reaction partners or their hydrolytic byproducts before the peptide is cleaved from the resin. Furthermore, phosphoramidate peptides containing non-oxidized Met could be potentially prepared utilizing the solid-phase approach.

We started our investigations of the Staudinger-phosphite reaction in solution by accessing a model aryl azidopeptide **3a** (Asp-Ala-Asp-Glu-Apa-Leu-Ile-Pro-Gln-Gln-Gly), which is an epidermal growth factor receptor fragment (988–998). Standard conditions for the diazotization of anilines, which can be incorporated into peptides with the commercially available Fmoc-Apa(Boc)-OH building block, utilizing $NaNO_2$ followed by the addition of $NaN_3$ under acidic conditions were applied to **3a** (Scheme 2). The corresponding aryl azidopeptide **1a** (Asp-Ala-Asp-Glu-Pap-Leu-Ile-Pro-Gln-Gln-Gly) was isolated by semi-preparative HPLC in 29% overall yield including SPPS. In comparison, the Fmoc-based SPPS route in analogy to Scheme 1A with commercially available Fmoc-Pap-OH delivered the same peptide **1a** in only 0.5% isolated yield. Aryl azidopeptide **1a** was then subjected to the Staudinger-phosphite reaction in solution in tris buffer at pH 8.2 with a water-soluble *o*-nitrobenzyl-phosphite [20], which delivered peptide **2a** bearing a caged phosphorylated tyrosine analog in 69% isolated yield. Furthermore, the application of the solid-phase strategy for the preparation of **2a** was unsuccessful due to an intrinsic light-sensitivity of this phosphoramidate.

After these solution-phase strategies for the acquisition and transformation of aryl azidopeptides **1**, we turned our attention to a solid-phase Staudinger-phosphite reaction (Scheme 1B). Since the Fmoc-based SPPS with an Fmoc-Pap-OH building block delivers only small quantities of aryl azidopeptides **1** (Scheme 1A), we aimed for a step-wise protocol, in which the peptide is reacted with phosphites directly after coupling of the Fmoc-Pap-OH building block. Afterwards, SPPS coupling cycles are continued and the final phosphoramidate peptide **2** is cleaved from the solid support by a standard TFA treatment. We first investigated the stability of phosphoramidate peptides **2** in the presence of piperidine or TFA. Phosphoramidate peptide **2b** (Phe[*p*-NHP(O)(OBu)$_2$]-Ala-Asp-Glu-Phe-Leu) was prepared by Fmoc-based SPPS of the corresponding azide precursor on a Wang resin, TFA cleavage and Staudinger-phosphite reaction in solution with tributylphosphite according to our previous report (Scheme 3A, Route II) [20]. Subsequent treatment either with 20% piperidine in DMF for 24 h or with 95% aqueous TFA at room temperature for 2 h caused no degradation of **2b** under these conditions. Since aryl phosphoramidates showed the necessary stability, we focused on performing the Staudinger-phosphite reaction on the solid support (Scheme 3A, Route I). A previously employed aryl azidopeptide **1b** (Pap-Ala-Asp-Glu-Phe-Leu) was incubated on the solid support with trimethylphosphite or tributylphosphite in DMSO to form immobilized phosphoramidate

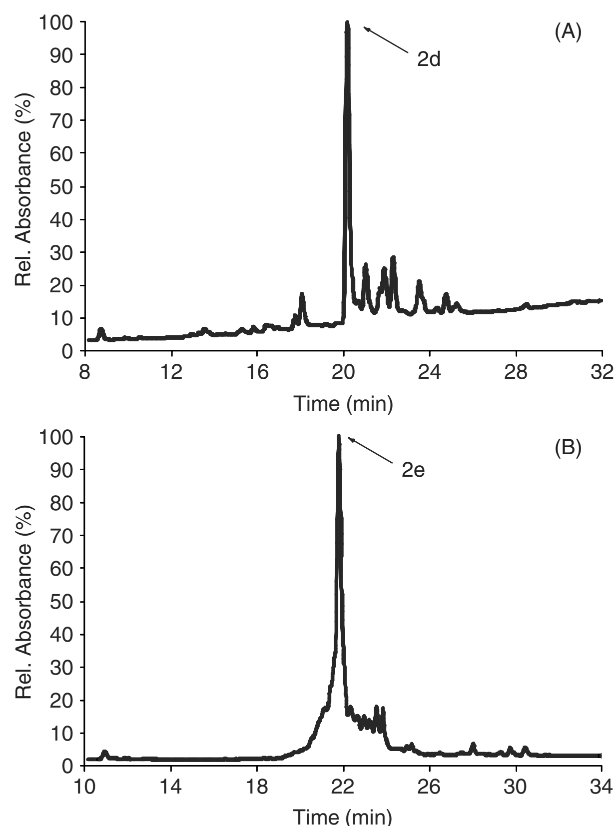


**Figure 1.** LC-UV$_{230\ nm}$ trace for crude peptides **2d** (A) and **2e** (B) released from the solid support.

peptides. Resins were washed with DMF and $CH_2Cl_2$, and peptides **2b** and **2c** (Phe[*p*-NHP(O)(OMe)$_2$]-Ala-Asp-Glu-Phe-Leu) were released from the solid support under the same acid-promoted cleavage conditions used during the stability measurement. LC-MS analysis of both crude mixtures indicated the presence of phosphoramidate peptides **2b** and **2c** as the only reaction products (Supporting Information). Encouraged by these results, we targeted phosphoramidate analogs of a phosphorylated adhesion and degranulation promoting adapter protein fragment (586–600), peptides **2d** (Cys-Arg-Pro-Ile-Glu-Asp-Asp-Gln-Glu-Val-Phe[*p*-NHP(O)(OMe)$_2$]-Asp-Asp-Val-Ala-Glu) and **2e** (Cys-Arg-Pro-Ile-Glu-Asp-Asp-Gln-Glu-Val-Phe{*p*-NHP(O)[OBn(2-$NO_2$)]$_2$}-Asp-Asp-Val-Ala-Glu), as a more challenging synthetic aim, in which the phosphoramidate is located more than ten amino acids away from the *N*-terminus (Scheme 3B). A rink amide resin bound aryl azidopeptide **1c** (Pap-Asp-Asp-Val-Ala-Glu) was incubated with trimethylphosphite or tris(2-nitrobenzyl) phosphite in DMF, followed by further amino acid couplings. Final TFA cleavage delivered full length peptides, **2d** and **2e**. HPLC and HRMS analysis for the crude peptide material indicated peptides, **2d** and **2e** as single major peaks (Figure 1), which were isolated by semi-preparative HPLC in 12% and 10% overall yield, respectively.

In summary, we have succeeded in the preparation of phosphoramidate-containing peptides from aryl azidopeptides, in which the phosphoramidate moiety can be placed distant from the peptide *N*-terminus. In order to synthesize these molecules, the Staudinger-phosphite reaction was performed both in solution and on the solid support, which delivered light-sensitive



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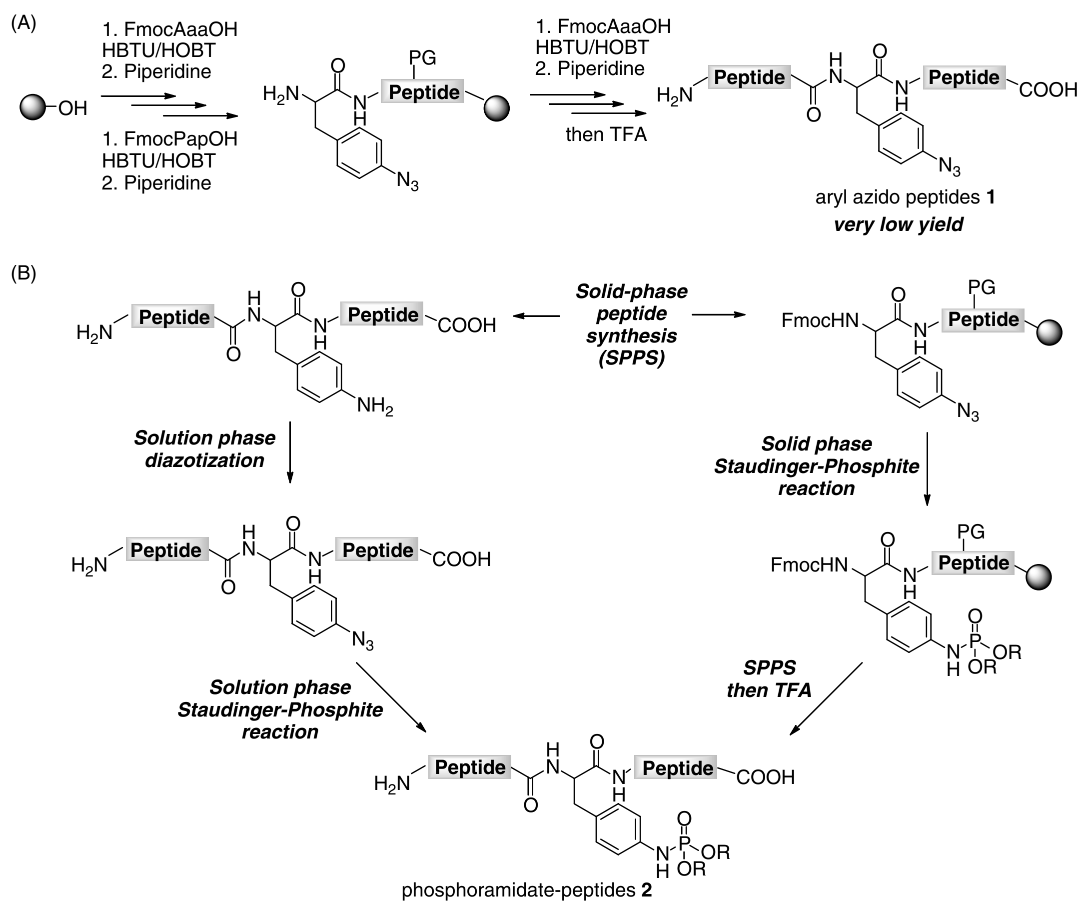
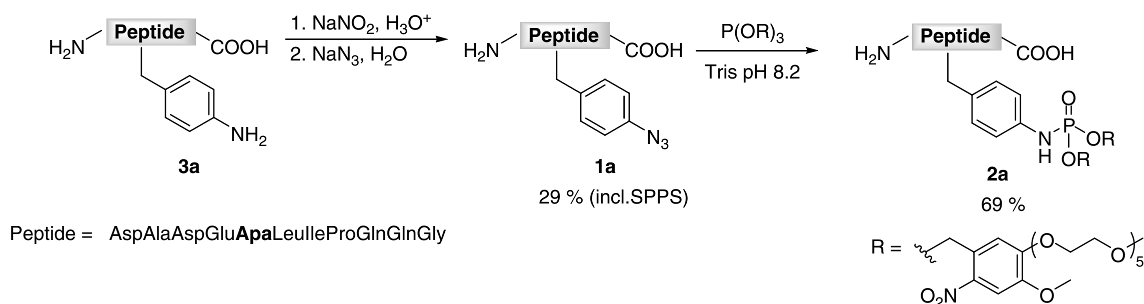



**Scheme 1.** (A) Fmoc-based SPPS for the incorporation of Pap. (B) Solution- and solid-phase strategies for the synthesis of phosphoramidate peptides.




**Scheme 2.** Diazotization of aniline peptide **3a** in solution to aryl azidopeptides **1a**, and subsequent Staudinger-phosphite transformation into photo-sensitive peptide phosphoramidate **2a**. Phe indicates the azide position.

analogs of phosphorylated peptides in high yields. Current investigation in our laboratory focuses on the development of diazotization protocols, which can be performed on the solid support, which allow a convergent synthesis of phosphoramidate peptides.

## Materials and Methods

Peptide syntheses were carried out with an automated peptide synthesizer ABI 433A (Applied Biosystems) utilizing standard Fmoc SPPS conditions (amide coupling: HBTU/HOBt/DIPEA; deprotection: 25% piperidine in DMF). All canonical and unnatural [Fmoc-Pap-OH, Fmoc-Apa(Boc)-OH] L-amino acid building blocks were purchased from Novabiochem or Bachem. LC-UV and LC-MS spectra were recorded on an Agilent 6210 TOF LC/MS system, Agilent Technologies, Santa Clara, CA, USA. Spray voltage was set to 4 kV. Drying gas flow rate was set to 25 psi. Separation of the sample was performed on a Luna C18(2) 100 A column (5 µm, 4.6 mm × 150 mm) at a flow rate of 0.6 ml/min. The following solvent (A = 1% AcOH in $H_2O$, B = 1% AcOH in MeCN) gradient was applied: 0% B 0–5 min; 0–60% B 5–25 min; 60% B 25–32 min. Preparative HPLC purification for peptides was performed on a JASCO LC-2000 Plus system using a Kromasil RP18 column (25 mm × 250 mm) at a flow rate of 16 ml/min. The following solvent (A = 0.1% TFA in $H_2O$, B = 0.1% TFA in MeCN) gradient was applied: 0% B 0–5 min; 0–60% B 5–55 min; 60–100% B 35–40 min; 100%



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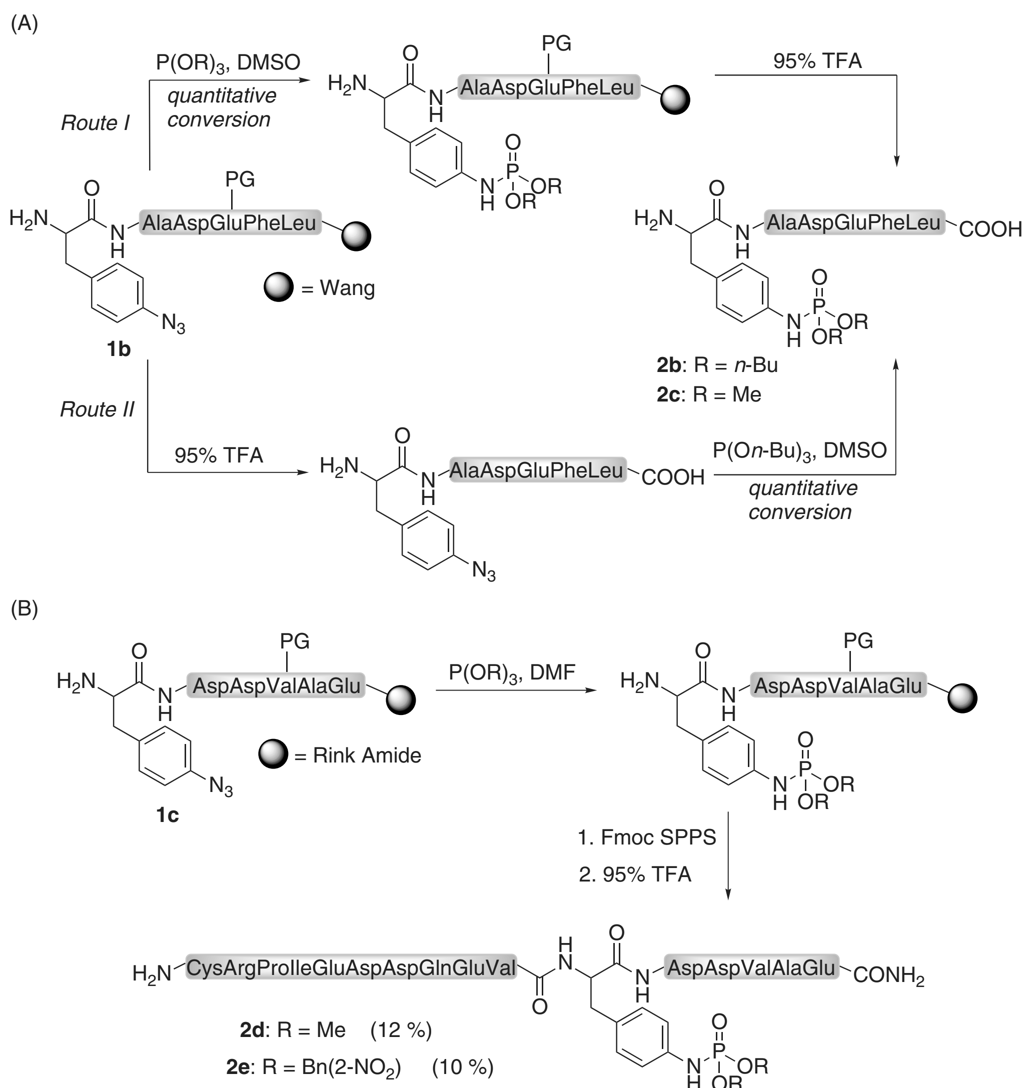


**Scheme 3.** (A) Preparation of peptide phosphoramidates in solution (Route II) and on the solid support (Route I). (B) Synthesis of peptide phosphoramidates **2d** and **2e** on the solid support.

B 40–50 min. Phosphoramidate peptide **2b**, tris(2-nitrobenzyl) phosphite and tris(4(2,5,8,11,14-pentaoxahexadecan-16-yloxy)-5-methoxy-2-nitrobenzyl) phosphite were prepared according to the literature protocols [20,34].

### Reactions of Resin Bound Peptides

Resin bound peptides **2b** and **2c** were prepared from **1b** (25 µmol) upon overnight incubation at RT in DMSO (1.5 ml) contained tributyl- and trimethylphosphite (125 µmol), respectively. Peptides **2b** and **2c** were cleaved from the resin to give white solids (24 mg, 90% crude and 21 mg, 86% crude, respectively), and they were analyzed by LC-UV, to show that desired phosphoramidates were the only detectable products (Supporting Information). Identities of products were confirmed by HRMS, **2b**: $m/z = 948.4369$ [M + H]$^+$, calcd.: 948.4532; **2c**: $m/z = 864.3625$ [M + H]$^+$, calcd.: 864.3593. Resin bound peptides **2d** and **2e** were prepared upon overnight incubation at RT of **1c** (50 µmol) in DMF with trimethyl- and tris(2-nitrobenzyl) phosphite (250 µmol), respectively, followed by standard Fmoc SPPS. Peptides **2d** and **2e** were cleaved from the resin, purified by semi-preparative HPLC and lyophilized to give white solids (12 mg, 12% and 11 mg, 10%, respectively); HRMS for **2d**: $m/z = 1001.4174$ [M + 2H]$^{2+}$, calcd.: 1001.4170; HRMS for **2e**: $m/z = 1122.4383$ [M + 2H]$^{2+}$, calcd.: 1122.4337.

### Reactions of Peptides in Solution

To a solution of crude peptide **3a** released from the resin (6.8 mg, 5.4 µmol) in 8 M HCl (300 µl) at 0 °C, $NaNO_2$ (0.7 mg, 10 µmol) in water (50 µl) was added. The solution was stirred for 15 min, followed by the addition of $NaN_3$ (1.1 mg, 15 µmol) in water (50 µl). After 15 min at 0 °C, the mixture was neutralized with aqueous NaOH (1 M), and peptide **1a** was isolated by preparative HPLC, followed by lyophilization to give a white solid (2.8 mg, 41% yield of the transformation in solution; 29% overall yield including SPPS). HRMS for **1a**: $m/z = 1273.5801$ [M + H]$^+$, calcd.: 1273.5808. Peptide **2a** (400 mg) was prepared from **1a** upon overnight incubation with tris(4-(2,5,8,11,14-pentaoxahexadecan-16-yloxy)-5-methoxy-2-nitrobenzyl) phosphite (8 mg, 5.5 µmol) in tris buffer (400 µl, 1 M, pH 8.2) at RT. Peptide **2a** was isolated from the mixture via preparative HPLC and lyophilized to give a white



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solid (0.45 mg, 69%). HRMS for **2a**: $m/z = 1079.4680$ $[M + 2H]^{2+}$, calcd.: 1079.4662.

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### Supporting information

Supporting information may be found in the online version of this article.

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