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Exploiting Azido-dichloro-Triazine as a Linker for Regioselective Incorporation of Peptides through their N, O, S Functional Groups

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Abstract

In the field of bioconjugation, linker development has witnessed massive growth in recent years. 2,4,6-Trichloro-1,3,5-triazine (TCT) is a tridentate linker that can accommodate three distinct nucleophiles. Herein, the reaction of azido triazine derivatives with nucleophiles (amine, thiol and phenol) is studied. The replacement of first chlorine was performed at 0 °C while that of the last chlorine was achieved successfully at rt. As a proof of concept of this strategy with potential application in biological studies, pentapeptides (Ac-XGGFL-NH₂ where X = Lys or Tyr or Cys) were reacted with 2-azido-4,6-dichlorotriazine to replace the first and second chlorine at 0 °C and at rt, respectively. The reactivity of 2-azido-4,6-dichlorotriazine was found to be similar for the α and ε amine group present in same peptide. These findings demonstrate the applicability of 2-azido-4,6-dichlorotriazine as a linker with potential further application in bioconjugation.

Introduction

In the field of bioconjugation, *LINKER* development has witnessed massive growth in recent years [1-4]. The linker is usually a spacer molecule that connects drugs to two or more biomolecules (drugs, peptides or proteins) of biological significance [4-6]. It must ensure the stability of a new molecule after bioconjugation without altering the biological significance of the biomolecules attached to it and must facilitate the controlled release of the drug/peptide at the target site [6-8]. Due to its low cost and ease of sequential incorporation of three distinct nucleophiles at different temperatures, 2,4,6-Trichloro-1,3,5-triazine (TCT) is frequently used as a building block in organic synthesis to access complex molecular architectures [9-11]. The three reactive chlorines present in TCT offers uniqueness, responsible for its efficient reactivity with nucleophiles. The incorporation of the first nucleophile into TCT is carried out at 0 °C, the second at room temperature (rt), and the third under heating or even reflux conditions, which cannot be considered biologically compatible (Figure 1).



Figure 1. Sequential incorporation of nucleophiles (Nu₁, Nu₂ and Nu₃)

The loss of σ -bond electron withdrawal (of a chlorine atom) and the increase in π -orbital electron density (electron-donating property of the incorporated nucleophile) on the triazine ring decreases the reactivity for further nucleophilic substitution reactions, and hence elevated temperatures are required for further reactions [9, 12-16]. In our earlier work, we demonstrated the concept of orthogonal chemoselectivity, which was defined as "discrimination between reactive sites in any order" using amine, thiol and alcohol/phenol to find the preferential order of incorporation onto TCT as a linker [14, 17]. However, the last replacement was achieved at a higher temperature [14]. To address this, and given the electron-withdrawing behavior of azide functionality [18], in another study we introduced azide as one of the nucleophiles, in addition to thiol, alcohol and phenol, onto TCT. Azide incorporation onto TCT rendered the last chlorine replacement at rt, thus being compatible with a biological context. In addition to the above, azide functionality also unfolds the

possibility of further transformation mainly *via* click chemistry to incorporate another chemical entity [19].

The idea of not incorporating amine in our previous work, as once amine is incorporated it's not possible for any other nucleophile to react with TCT and hence posing a limitation for triorthogonal chemoselectivity substitution. However, the amine-bearing triazine motif is the "Master Key" as it is integral part of several commercial molecules [20]. Therefore, in the present study, we examined the TCT core with three possible substituents, where one is occupied by azide and another by amine. The third substituent could be any nucleophile *viz.*, amine, thiol, alcohol or phenol. Also, as an application in biological systems, we also report the reactivity of TCT using peptides bearing Lys, Cys and Tyr, which contain amine, thiol and phenol nucleophiles, respectively, and mimics biological systems.

Result and Discussion

The first target of the present study was to explore the possible combinations in the sequential order of incorporating different nucleophiles to obtain the triazine core, where one substitution is azide (facilitating all process and/or further modification through e.g. Click chemistry), another one amine (due to its importance in biological systems) and the third alcohol or thiol or phenol or again amine. **Figure 2** shows the nucleophiles used in this part of the study. Through



Figure 2. Nucleophiles chosen for synthesizing tri-substituted triazine. Through the whole text, the following color code has been used: dark blue for azide, blue for amine, red for alcohol, pink for phenol, and green for thiol

All the possible combinations to synthesize tri-substituted TCT, under the previous indicated premises $-N_3$ and amine substitution are shown in **Scheme 1**. Azide was evaluated at all three positions. TCT-N₃ (1) was synthesized as described earlier by our group [18]. For the second incorporation, amine, thiol, alcohol and phenol were reacted with TCT-N₃ at 0 °C (-20 °C for phenol) for 30 mins. Successful incorporation was observed in case of amine, thiol and phenol, affording the formation of **6**, **7** and **8**. However, alcohol yielded no product, as demonstrated earlier [18]. The use of a lower temperature for the case of phenol is due to the deactivating effect of the phenol, which once incorporated facilitates the incorporation of a second molecule [17].

The di-substituted derivatives (6, 7 and 8) were further treated with amine for the third substitution. The reaction was performed at rt for 3 h (18 h in case of 6). Successful incorporation of amine onto the di-substituted derivatives was observed in all cases affording 14, 15 and 16. The incorporation of a second amine when an amine is already present (6) is possible but more demanding than other cases.



Scheme 1. Nucleophilic substitution reactions carried out in this study.

To evaluate azide at the second position, mono-substituted TCTs (m-TCTs) (2, 3, 4 and 5) were synthesized successfully using amine, alcohol, thiol, and phenol, respectively, and TCT at 0 °C (-20 °C in case of phenol) for 30 mins. m-TCTs were reacted with sodium azide at 0 °C for 30 mins to form disubstituted TCT (d-TCT) derivatives (6, 10, 7 and 8, respectively) with full conversion in all cases. d-TCTs were reacted further with amine at rt for 3 h for the third substitution. Full conversion of 6, 7, and 8 to the tri-substituted TCT (14, 15 and 16, respectively) was observed. However, the reaction of 10 (alcohol substituent) with amine at rt for 16 h rendered low conversion as monitored by TLC. Then, the reaction was heated at 40 °C for 16 h to completion, affording 17.

To evaluate azide substitution at the third position, m-TCTs (2, 3, 4 and 5) were reacted with amine at rt for 2 h. All the reactions afforded d-TCTs (9, 11, 12 and 13, respectively). Finally, compounds 9, 11, 12 and 13 were dissolved in acetone and reacted with sodium azide in water at 40 °C for 2 days. Product formation was only observed in 12 and 13, affording 15 and 16, respectively. Summary of all these experiments are shown in Table 1.

Order*			Compound				Yield
1 st	2 nd	3 rd	14	15	16	17	(%)
N ₃	NH	NH	\checkmark			\bigcirc	91%
N_3	SH	NH		1			94%
N_3	pOH**	NH			\checkmark		91%
N_3	ОН	NH				\checkmark	90%
NH	N ₃	NH	~				91%
NH	NH	N ₃	n.r.				-
SH	N ₃	NH		\checkmark			93%
SH	NH	N ₃		\checkmark			93%
рОН	N ₃	NH			\checkmark		91%
рОН	NH	N ₃			\checkmark		90%
ОН	N ₃	NH				\checkmark	90%
ОН	NH	N_3				n.r.	-

Table 1: Reactivity of TCT with nucleophiles.

*Indicates the order of nucleophile incorporation onto TCT; **pOH = Phenolic

 \checkmark = product obtained; n.r. = no reaction

In addition to these reactions, a competitive experiment was conducted at each substitution level to prove the selectivity of amine as nucleophile in the presence of other nucleophiles in the same reaction mixture (**Scheme 2**, **Table 2**). Thus, TCT-N₃ (1 equiv) was simultaneously treated with amine, thiol, alcohol and phenol (1 eq. each) in one pot in order to shed light on the competition between nucleophiles for the formation of product. The reaction was stirred at 0 °C for 30 mins

and afforded only the amine derivative (6) as demonstrated by TLC and confirmed by HPLC. Even phenol did not react with TCT-N₃ in the presence of amine in the same reaction.

A competitive experiment was also conducted for each d-TCT, which afforded only amine substituted derivatives (Table 2), as expected from our earlier results. The idea of a competitive experiment is crucial in bioconjugation, as most peptides/antibodies have amino acids with side chain functional groups. Here we demonstrate the selectivity of amine towards nucleophilic substitution reaction onto TCT derivatives, affording only one product.



Scheme 2. Competitive reaction carried out on TCT-N₃ (1) and d-TCTs (6, 7, 8 and 10) with azide functionality

|--|

Compound	Position 1*	Position 2*	One pot addition	Product obtained
2	N_3	-	NH+SH+OH+pOH	Only 6
6	N_3	NH	NH+SH+OH+pOH	Only 14
7	N_3	SH	NH+SH+OH+pOH	Only 15
8	N_3	рОН	NH+SH+OH+pOH	Only 16
10	N_3	OH	NH+SH+OH+pOH	Only 17

*Similar result when position 1 and position 2 interchanged

To understand the electronic effect of the charges carried by chlorine present on TCT, DFT calculations were performed. Geometry optimization using the density functional theory (DFT) was performed in the gas phase using Gaussian09 program package, employing the B3LYP (Becke three parameters Lee–Yang–Parr exchange correlation functional) and the 6-311G++(d,p) as basis set. Geometry optimization was followed by frequency calculations until no negative eigen values were obtained. The optimized geometries were used to perform natural bond orbital (NBO) calculations to determine the atomic charges on chlorine present in the molecule (**Table 3**).

Common d	Natural atomic charges on Cl				
Compound	Third Cl	Third Cl Second Cl			
ТСТ	0.088	0.088	0.088		
1	-	0.078	0.077		
2	-	0.049	0.049		
3	-	0.065	0.061		
4	-	0.068	0.062		
5	-	0.069	0.069		
6	-	<u>-</u>	0.038		
7	-	-	0.051		
8	-0-	-	0.058		
9	-	-	0.010		
10	-	-	0.051		
11	-	-	0.024		
12	-	-	0.025		
13	-	-	0.031		

Table 3: Charges carried by chlorine in TCT, m-TCTs and d-TCTs.

TCT carried more charge on chlorine compared to m-TCTs, thereby explaining its greater reactivity (**Table 3**). Among m-TCTs, the reduction of charge was less in case of **1** (from 0.088 to 0.078) compared to **2**, **3**, **4** and **5** (0.049 to 0.069). This observation could be explained by the substitution of azide (electron withdrawing) in the case of **1** compared to that of other nucleophiles (**2**, **3**, **4** and **5**) which are electron-donating. Since **1** had a high charge on chlorine, the second substitution at 0 °C can be explained. Unlike **1** (0.078), the lower charge carried by "CI" in **2**

(0.049), **3** (0.065), **4** (0.068) and **5** (0.069) allowed the reaction to be performed at rt. Among the d-TCTs, the charges carried by third chlorine were slightly higher when azide was one of the substituents compared to the rest. **6** (0.038) carried a smaller charge than **7** (0.051), **8** (0.058) and **10** (0.051). In the case where azide was the third substituent (**9**, **11**, **12** and **13**), the charge carried by chlorine decreased dramatically. This observation may explain longer reaction times in these cases. For **9** and **11**, no product formation was observed as the charge on chlorine was significantly lower, hence deactivating the ring towards the azide substitution under the given reaction conditions.

From the first part of this study, we can conclude that the introduction of the azide in first position is very beneficial for the two-remaining position. Thus, to expand the applicability of the TCT-N₃ (1) core in a more biological context peptides were used as nucleophiles. For this purpose, pentapeptides (Ac-X-GGFL-NH₂), synthesized using SPPS, where X = Lys, Tyr or Cys, were sequentially incorporated to the TCT-N₃ (Scheme 3). Ser was not attempted due to the poor reactivity of the alcohol as demonstrated in the first part of this study and reported previously [14, 18]. The reactivity of the pentapeptides was studied with TCT-N₃ (1) using K₂CO₃ as base in ACN/water mixture as solvent in contrast of the model studies where ethyl acetate was used (Scheme 3).



Scheme 3. Reaction of Lys, Cys, Tyr containing pentapeptides with TCT-N₃. Amino acid structures represent L-configuration

An equimolar solution of **1** and Ac-XGGFL-NH₂ at 0 °C in the presence of excess K_2CO_3 (pH > 8) in ACN/water mixture as solvent was reacted for 30 mins. Completion of the reaction was monitored by HPLC in all cases (Figure 3). The reaction mixture was extracted using ethyl acetate (to remove unreacted **1**) to afford pure TCT-N₃-Lys, TCT-N₃-Tyr and TCT-N₃-Cys.



Figure 3. HPLC chromatogram of the TCT-N₃-Peptide conjugate [5-95 % of CH₃CN (0.1% TFA/ H₂O (0.1 %TFA) over 15 mins]

Based on our earlier findings, once an amine is incorporated on triazine ring, only another amine can undergo reaction for the replacement of the last chlorine on the triazine core [14, 18]. Hence, aqueous solution of TCT-N₃-Lys was collected and another equivalent of Ac-KGGFL-NH₂ (Lyspeptide) was added, followed by addition of a K_2CO_3 solution. This mixture was then stirred at rt for 4 days to form TCT-N₃-Lys-Lys. The reaction was monitored by HPLC (Figure 4).



Figure 4. HPLC chromatogram of TCT-N₃-Lys-Lys synthesis [5-95 % of CH₃CN (0.1% TFA/ H₂O (0.1 %TFA) over 15 mins]

However, when the TCT-N₃ is substituted with Tyr and Cys peptides, the possibilities are broadening. TCT-N₃-Tyr was reacted in parallel with Tyr-peptide and Lys-peptide in the presence of K_3CO_3 using water as solvent at rt to replace the last chlorine. The reaction with Lys-peptide was completed in 4 h and reaction with Tyr-peptide in 8 h (Figure 5). Similarly, TCT-N₃-Cys was reacted in parallel with Lys-peptide and Tyr-peptide at rt in the presence of K_2CO_3 using water as solvent for 2 days until the HPLC showed complete consumption of reactants (Figure 6). These reactions yielded TCT-N₃-Cys-Lys and TCT-N₃-Cys-Tyr, respectively.



Figure 5. HPLC chromatogram of TCT-N₃-Tyr-Tyr and TCT-N₃-Tyr-Lys synthesis [20-70 % of CH₃CN (0.1% TFA/ H₂O (0.1 %TFA) over 15 mins]



Figure 6. HPLC chromatogram of TCT-N₃-Cys-Tyr and TCT-N₃-Cys-Lys synthesis [5-95 % of CH₃CN (0.1% TFA/ H_2O (0.1 %TFA) over 15 mins]

Peptides/proteins usually hold two different amines (in terms of reactivity) (α -NH₂ in amino acids and ϵ -NH₂ in Lys side chain). To check the reactivity of **1** against these two amino groups, another pentapeptide (H-KGGFL-NH₂) was synthesized. This peptide, which bears free both α -NH₂ and ϵ -NH₂ functions, was reacted with **1** (3 eq.) in the presence of K₂CO₃ as base at 0 °C for 30 mins. Although in principle the α -NH₂ is less reactive than the ϵ -NH₂, HPLC monitoring demonstrated similar reactivity of **1** towards the two amines, as double incorporation was observed. Excess of **1** was easily removed by washing reaction mixture with ethyl acetate, thus affording the product in aqueous layer (Figure 7).



Figure 7. Reaction of 1 with α and ϵ -NH₂ in Lys in pentapeptide (H-KGGFL-NH₂) [5-95 % of CH₃CN (0.1% TFA/ H₂O (0.1 %TFA) over 15 mins]

Conclusions

In our current efforts to develop biologically compatible linkers, azido triazine derivatives emerge as useful tool for bioconjugation. Thus, we have demonstrated that the first chlorine in azidodichloro-*s*-triazine (1) can be easily replaced with an amino, phenol or thiol compound at 0 °C and the second one at rt. However, if amine is introduced first, only a second amine can be incorporated at rt as no other nucleophiles can be accommodated. If phenol or thiol is introduced in 1, amine can easily replace the last chlorine at rt, a condition compatible with biological systems. In addition, amine as nucleophile is highly selective upon reaction with 1 in the presence of other nucleophiles like phenol and thiol.

We exploited this selectivity further for the reaction of pentapeptides (Ac-XGGFL-NH₂ where X = Lys or Tyr or Cys) with 1 in the presence of K₂CO₃ as models of other biological systems. We demonstrate the incorporation of Lys- or Tyr- or Cys-bearing pentapeptide onto 1 at 0 °C and then Tyr- and Lys-peptide at rt using aqueous solvents under mild conditions. The reactivity of 1 was similar towards the α and ε amine group present in the peptide. Based on our results, which support the applicability of azido-dichloro-triazine (1), we propose that this molecule can be further exploited as a linker in bioconjugation involving peptides/proteins

Experimental

General

All the chemicals and diisopropylethylamine were purchased from Sigma-Aldrich (Sigma-Aldrich, Germany). Fmoc-L-amino acids (from IRIS, Germany) were used for Solid Phase Peptide Synthesis (SPPS). The solvents used were of analytical and HPLC reagent grade. Magnetic resonance spectra (¹H and ¹³C) were recorded with Bruker 400 MHz spectrometer (Bruker, Billerica, MA, USA), and chemical shift values are reported in δ units (ppm) using TMS as internal standard. Follow-up of the reactions and checks of the purity of the compounds were done by TLC on silica-gel-protected aluminum sheets 60 F254 (Merck, Germany), and the spots were detected by exposure to UV light at $\lambda = 254$ nm. Analytical HPLC was performed on an Agilent 1100 system using a Phenomenax C18 column (3µm, 4.6×50 mm) by dissolving the sample in CH₃CN only. Chemstation software was used for data processing. Buffer A: 0.1% TFA in H₂O, buffer B: 0.1% TFA in CH₃CN were used in HPLC. High resolution mass spectrometry (HRMS) was

performed using a Bruker ESI-QTOF mass spectrometer (Bruker, Billerica, MA, USA) in positiveion mode.

Synthetic protocol

Synthesis of 2-azido-4,6-dichloro-1,3,5-triazine (1)

The procedure for the synthesis of TCT-N₃ was followed as explained in our earlier work [18].

Synthesis of mono nucleophilic substituted derivatives

TCT (0.27 mmol) was dissolved in EtOAc (1 mL) and cooled to 0 0 C for 5 min. Nucleophile (0.27 mmol) was then added to the above stirring solution, followed by addition of DIEA (0.27 mmol). The reaction was stirred at 0 0 C (-20 0 C in the case of phenol) for 30 min. The progress of the reaction was monitored by TLC (EtOAc/hexane (1:4) as mobile phase), until no starting material was observed. The solution was washed several times with water to remove DIEA salts. The organic layer was collected, dried over MgSO₄, filtered and concentrated to afford pure product, which was used for the next step without further purification. Compounds **3**, **4** and **5** have been reported in our earlier publication [18].

4,6-dichloro-N-isopentyl-1,3,5-triazin-2-amine (2)

Yield = 61 mg (96 %); Off-white semi-solid; HPLC [30-95 % of CH₃CN (0.1% TFA/ H₂O (0.1 % TFA) over 15 mins] t_R = 7.7 min; ¹H NMR (400 MHz, CDCl₃): 0.88 (d, J = 6.6 Hz, -CH₃, 6H), 1.40 (m, -CH₂, 2H), 1.59 (m, -CH, 1H), 3.42 (m, -CH₂, 1H), 5.70 (brs, -NH, 1H); ¹³C NMR (100 MHz, CDCl₃): 22.4, 25.7, 37.9, 39.9, 165.8, 171.0; LCMS m/z: calcd. for C₈H₁₂Cl₂N₄: [M+H]⁺ 235.05, [M+CH₃CN+H]⁺ 278.07; found: 235.12 [M+H]⁺, 278.16 [M+CH₃CN+H]⁺.

Second substitution in the case of mono-azide TCT derivative

TCT-N₃ (0.27 mmol) was dissolved in EtOAc (1 mL) and cooled to 0 $^{\circ}$ C for 5 min. Nucleophile (0.27 mmol) was then added to the above stirring solution, followed by addition of DIEA (0.27 mmol). The reaction was stirred at 0 $^{\circ}$ C (-20 $^{\circ}$ C in the case of phenol) for 30 min. The progress of the reaction was monitored by TLC (EtOAc/hexane (1:4) as mobile phase) until no starting material was observed. The solution was washed several times with water to remove DIEA salts. The organic layer was collected, dried over MgSO₄, filtered and concentrated to afford pure

product, which was used for the next step without further purification. Compounds 7 and 8 have been reported in our earlier publication [18].

4-azido-6-chloro-N-isopentyl-1,3,5-triazin-2-amine (6)

Yield = 61 mg (94 %); Off-white semi-solid; HPLC [30-95 % of CH₃CN (0.1% TFA/ H₂O (0.1 % TFA) over 15 mins] t_R = 8.2 min; ¹H NMR (400 MHz, CDCl₃): 0.94 (d, J = 6.6 Hz, -CH₃, 6H), 1.50 (m, -CH₂, 2H), 1.64 (m, -CH, 1H), 3.49 (m, -CH₂, 2H), 6.59 (brs, -NH, 1H); ¹³C NMR (100 MHz, CDCl₃): 22.4, 25.7, 37.9, 39.6, 166.2, 170.2. HRMS: m/z: calcd. for C₈H₁₂ClN₇: [M+H]⁺ 242.0915; found: 242.0926.

Second substitution in the case of m-TCT derivatives

Isopentyl amine (0.27 mmol) was added to m-TCT (0.27 mmol) in EtOAc (1 mL), followed by addition of DIEA (0.27 mmol). The reaction was stirred at room temperature for 3 h (30 mins at 0 ^oC in the case of azide and overnight for alcohol). The progress of the reaction was monitored by TLC (EtOAc/hexane (1:4) as mobile phase) until no starting material was observed. The solution was concentrated to dryness and the residue was dissolved in DCM and washed several times with water to remove DIEA salts. The organic layer was collected, dried over MgSO₄, filtered and concentrated to afford pure product, which was used for the next step without further purification. **10** and **12** have been reported in our earlier publication [17, 18].

6-chloro- N^2 , N^4 -diisopentyl-1, 3, 5-triazine-2, 4-diamine (9)

Yield = 69 mg (89 %); Off-white semi-solid; HPLC [30-95 % of CH₃CN (0.1% TFA/ H₂O (0.1 % TFA) over 15 mins] t_R = 10.4 min; ¹H NMR (400 MHz, CDCl₃): 0.95 (d, J = 6.6 Hz, -CH₃, 12H), 1.49 (m, -CH₂, 4H), 1.66 (m, -CH, 2H), 3.49 (q, J = 8.2 Hz, -CH₂, 4H), 5.79 (brs, -NH, 2H); ¹³C NMR (100 MHz, CDCl₃): 22.2, 24.6, 36.8, 69.0, 170.9, 172.3. HRMS: m/z: calcd. for C₁₃H₂₄ClN₅: [M+H]⁺ 286.1799; found: 286.1815.

4-chloro-N-isopentyl-6-(isopentyloxy)-1,3,5-triazin-2-amine (11)

Yield = 67 mg (87 %); Off-white semi-solid; HPLC [30-95 % of CH₃CN (0.1% TFA/ H₂O (0.1 % TFA) over 15 mins] t_R = 10.5 min; ¹H NMR (400 MHz, CDCl₃): 0.92 (d, J = 6.5 Hz, -CH₃, 12H), 1.44 (m, -CH₂, 2H), 1.64 (m, -CH, 1H), 1.71 (m, -CH, 2H), 1.80 (m, -CH₂, 2H), 3.41 (m, -CH₂, 2H), 4.28 (m, -CH₂, 1H), 5.08 (m, -CH₂, 1H); ¹³C NMR (100 MHz, CDCl₃): 22.4, 22.5, 24.8, 25.7,

37.7, 38.7, 39.0, 65.1, 166.9, 167.3, 188.0. HRMS: m/z: calcd. for C₁₃H₂₃ClN₄: [M+H]⁺287.1653; found: 287.1667.

4-chloro-N-isopentyl-6-phenoxy-1,3,5-triazin-2-amine (13)

Yield = 73 mg (93 %); Off-white semi-solid; ¹H NMR (400 MHz, CDCl₃): 0.91 (d, J = 6.5 Hz, - CH₃, 6H), 1.63 (m, -CH₂, 2H), 1.69 (m, -CH, 1H), 4.43 (m, -CH₂, 2H), 7.17 (m, Ar-H, 2H), 7.28 (m, Ar-H, 1H), 7.43 (m, Ar-H, 2H); ¹³C NMR (100 MHz, CDCl₃): 22.4, 24.7, 30.9, 36.9, 121.3, 126.4, 129.7, 151.5, 172.2, 172.4, 173.2. HRMS: m/z: calcd. for C₁₄H₁₇ClN₄O: [M+H]⁺293.1163; found: 293.1176.

Tri-substitution in the case of d-TCT derivatives

Nucleophile (0.26 mmol) was added to a stirring solution of d-TCT (0.26 mmol) in EtOAc (1 mL), followed by addition of DIEA (0.26 mmol). The reaction was stirred at room temperature (40 ^oC in the case of alcohol as substituent) for 16 h. The progress of the reaction was monitored by TLC (EtOAc/hexane (1:5) as mobile phase) until no starting material was observed. The solution was washed several times with water to remove DIEA salts. The organic layer was collected, dried over MgSO₄, filtered and concentrated to afford pure product.

6-azido-N²,N⁴-diisopentyl-1,3,5-triazine-2,4-diamine (14)

Yield = 72 mg (91 %); Off-white semi-solid; HPLC [30-95 % of CH₃CN (0.1% TFA/ H₂O (0.1 % TFA) over 15 mins] t_R = 8.2 min; ¹H NMR (400 MHz, CDCl₃): 0.93 (d, *J* = 6.4 Hz, -CH₃, 12H), 1.45 (m, -CH₂, 4H), 1.66 (m, -CH, 2H), 3.41 (m, -CH₂, 4H), 5.19 (brs, -NH, 1H), 5.19 (brs, -NH, 1H); ¹³C NMR (100 MHz, CDCl₃): 22.5, 25.7, 29.7, 39.1, 166.5, 168.1. HRMS: m/z: calcd. for C₁₃H₂₄N₈: [M+H]⁺ 293.2197; found: 293.2206.

4-azido-N-isopentyl-6-(isopentylthio)-1,3,5-triazin-2-amine (15)

Yield = 78 mg (94 %); Off-white semi-solid; HPLC [30-95 % of CH₃CN (0.1% TFA/ H₂O (0.1 % TFA) over 15 mins] t_R = 12.4 min; ¹H NMR (400 MHz, CDCl₃): 0.94 (d, J = 6.5 Hz, -CH₃, 12H), 1.47 (m, -CH₂, 2H), 1.58 (m, -CH, 1H), 1.65 (m, -CH₂, 2H), 1.73 (m, -CH, 1H), 3.09 (m, -CH₂, 2H), 3.45 (m, -CH₂, 2H), 5.60 (brs, -NH, 1H); ¹³C NMR (100 MHz, CDCl₃): 22.3, 25.7, 27.6, 27.7, 28.2, 28.4, 38.3, 39.4, 164.8, 168.2, 183.0. HRMS: m/z: calcd. for C₁₃H₂₃N₇S: [M+H]⁺ 310.1828; found: 310.1836.

4-azido-N-isopentyl-6-phenoxy-1,3,5-triazin-2-amine (16)

Yield = 73 mg (91 %); Off-white semi-solid; HPLC [30-95 % of CH₃CN (0.1% TFA/ H₂O (0.1 % TFA) over 15 mins] t_R = 8.9 min; ¹H NMR (400 MHz, CDCl₃): 0.92 (d, *J* = 6.5 Hz, -CH₃, 6H), 1.43 (m, -CH₂, 2H), 1.63 (m, -CH, 1H), 3.41 (m, -CH₂, 2H), 5.96 (brs, -NH, 1H), 7.16 (m, Ar-H, 2H), 7.25 (m, Ar-H, 1H), 7.39 (m, Ar-H, 2H); ¹³C NMR (100 MHz, CDCl₃): 22.4, 25.6, 38.1, 39.4, 121.8, 125.7, 129.4, 151.9, 167.7, 170.4, 171.7. HRMS: m/z: calcd. for C₁₄H₁₇N₇O: [M+H]⁺ 300.1567; found: 300.1588.

4-azido-N-isopentyl-6-(isopentyloxy)-1,3,5-triazin-2-amine (17)

Yield = 71 mg (90 %); Off-white semi-solid; HPLC [30-95 % of CH₃CN (0.1% TFA/ H₂O (0.1 % TFA) over 15 mins] t_R = 10.6 min; ¹H NMR (400 MHz, CDCl₃): 0.94 (d, J = 6.3 Hz, -CH₃, 12H), 1.48 (m, -CH₂, 2H), 1.62 (m, -CH, 1H), 1.68 (m, -CH₂, 2H), 1.81 (m, -CH, 1H), 3.48 (m, -CH₂, 2H), 4.33 (m, -CH₂, 1H), 4.41 (m, -CH₂, 1H), 6.00 (brs, -NH, 1H); ¹³C NMR (100 MHz, CDCl₃): 22.4, 22.5, 24.8, 25.7, 37.4, 38.2, 39.3, 66.4, 167.6, 169.8, 171.6. HRMS: m/z: calcd. for C₁₃H₂₃N₇O: [M+H]⁺ 294.2053; found: 294.2069.

Synthesis of pentapeptide (Ac-XGGFL-NH₂ and H-KGGFL-NH₂)

SPPS was carried out manually in plastic syringe fitted with polyethylene porous using Fmoc/*t*Bu methodology. Fmoc-Rink-Amide AM resin (0.74 mmol/g) was washed with DMF (2×5 min). Deprotection of the Fmoc group was achieved by treatment of the resin with 20% piperidine/DMF (1×1 min and 1×7 min) followed by washing with DMF. The protected Fmoc-amino acids (3 eq.) were incorporated using DIC (3 eq.) and OxymaPure (3 eq.) in DMF, as a coupling system, 30 mins at rt. This was repeated until the tetrapeptide (H-GGFL-NH₂) was achieved. Washes between couplings and deprotections were performed with DMF (3×1 min). The tetrapeptidyl resin was divided into four parts of which two parts were coupled with Fmoc-Lys(Boc)-OH, one part coupled with Fmoc-Cys(Trt)-OH and last one with Fmoc-Tyr(*t*Bu)-OH using above coupling condition. Fmoc was removed in all the four cases followed by washing. Pentapeptides (H-KGGFL-NH₂, H-CGGFL-NH₂ and H-YGGFL-NH₂) were acetylated (acetic anhydride and DIEA in 1:2 ratio using DMF as solvent) for 30 mins. All the peptides were dried and cleaved from resin by treating with TFA/TIS/H₂O (95:2.5:2.5) for 1 h at rt. The cleavage mixture was evaporated with a stream of nitrogen, precipitated with Et₂O, centrifuged to afford desired peptide as confirmed by HPLC and LCMS (Table 4).

Synthesis of mono-substituted TCT-N₃ with Ac-XGGFL-NH₂

A stock solution of TCT-N₃ (10 mg/mL in ACN), Ac-XGGFL-NH₂ (5 mg/mL in water) and 100 mM aqueous K_2CO_3 solution was prepared. 1.1 eq of TCT-N₃ was cooled to 0 °C. To the stirring solution of TCT-N₃ was added 1 eq of peptide solution followed by 5 eq of K_2CO_3 solution (pH > 8). The reaction was stirred at 0 °C for 30 mins. The progress of the reaction was monitored by HPLC. After the completion of reaction, reaction mixture was washed with ethyl acetate to remove excess unreacted TCT-N₃ to afford TCT-N₃-Lys, TCT-N₃-Tyr and TCT-N₃-Cys in aqueous layer. The products were confirmed by LCMS (Table 4).

Synthesis of di-substituted TCT-N₃ with Ac-XGGFL-NH₂

To the mono-substituted TCT-N₃ (1 eq each of TCT-N₃-Lys, TCT-N₃-Tyr and TCT-N₃-Cys) was added 1 eq of Ac-XGGFL-NH₂ with 5 eq of K₂CO₃. The reaction was stirred at rt. The progress of reaction was monitored by HPLC. The reaction time was found to vary in each case. The reaction for synthesis of TCT-N₃-Lys-Lys was found to complete in 4 days. TCT-N₃-Tyr-Tyr and TCT-N₃-Tyr-Lys was found to be complete in 8 h and 4 h, respectively. TCT-N₃-Cys-Tyr and TCT-N₃-Cys-Lys were formed in 2 days. All the di-substituted TCT-N₃ were confirmed by LCMS (Table 4).

Reaction of TCT-N₃ and H-KGGFL-NH₂

3 eq of TCT-N₃ was cooled to 0 °C. To the stirring solution of TCT-N₃ was added 1 eq of H-KGGFL-NH₂ solution followed by 5 eq of K₂CO₃ solution (pH > 8). The reaction was stirred at 0 °C for 30 mins. The progress of the reaction was monitored by HPLC. After the completion of reaction, reaction mixture was washed with ethyl acetate to remove excess unreacted TCT-N₃ to afford TCT-N₃-K(TCT-N₃)-GGFL-NH₂ in aqueous layer. The product was confirmed by LCMS (Table 4).

Sl. No	Compound	HPLC (<i>t_R</i> in min)	Expected Mass M+[H] ⁺	Found Mass	% Conversion
1	Ac-KGGFL-NH ₂	5.6	562.69	562.37#, 282.01##	-
2	Ac-YGGFL-NH ₂	6.9	597.69	597.35 [#]	-
3	Ac-CGGFL-NH ₂	6.5	537.65	537.24#	-

Table 4. HPLC (5-95% B into A in 15 mins) and LCMS for peptide conjugates with TCT-N₃.

4	H-KGGFL-NH ₂	5.0	520.65	520.39#, 260.95##	-
5	TCT-N ₃ -Lys	8.2	716.20	716.21#, 358.96##	100
6	TCT-N ₃ -Tyr	9.1*	751.20	751.28#	100
7	TCT-N ₃ -Cys	8.1	691.17	691.24 [#]	100
8	TCT-N ₃ -Lys-Lys	8.1	1241.43	621.28##, 427.68###	100
9	TCT-N ₃ -Tyr-Lys	8.3*	1276.43	639.09##	100
10	TCT-N ₃ -Tyr-Tyr	8.9*	1311.43	656.78##	100
11	TCT-N ₃ -Cys-Lys	8.1	1216.39	609.07##	100
12	TCT-N ₃ -Cys-Tyr	8.5	1251.39	626.31##	100
13	TCT-N ₃ -K(TCT- N ₃)-GGFL-NH ₂	9.6	828.68	828.36#, 414.73##	100

*20-70% of B into A for 15 mins; #M+[H]+, ##[M+2H]+/2, ###[M+3H]+/3

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August 3, 2020

Dear Professor Distefano,

It is a pleasure for us to send you the manuscript entitled "Exploiting Azido-diChloro-Triazine as a Linker for Regioselective Incorporation of Peptides through their N, O, S functional groups" by Anamika Sharma, Ashish Kumar, Ayman El-Faham, Beatriz G. de la Torre, and Fernando Albericio for your consideration for publication as a full paper in *Bioorganic Chemistry*.

HIGHLIGHTS

- 1,3,5-Trichlorotriazine (TCT) is a tridentate linker that can accommodate three distinct nucleophiles.
- Azido triazine facilitates the incorporation of different nucleophiles (amine, thiol and phenol).
- The replacement of first chlorine was performed at 0 °C while that of the last chlorine was achieved successfully at rt.
- The proof of concept of this strategy for biological studies has been carried out by incorporation of pentapeptides (Ac-XGGFL-NH2 where X = Lys or Tyr or Cys)
- The azido-dichloro triazine has replaced the first and second chlorine at 0 °C and at rt, respectively.
- The reactivity of azido-dichloro triazine was found to be similar for the α and ϵ amine group present in same peptide.
- These findings demonstrate the applicability of azido-dichloro triazine as a linker with potential further application in bioconjugation.

Exploiting Azido-diChloro-Triazine as a Linker for Regioselective Incorporation of Peptides through their N, O, S functional groups

