**Introduction**

The selective and efficient labeling of biomolecules under physiological conditions is still hard to achieve with traditional biochemical or molecular biology tools. Since bioorthogonal chemical reactions can be performed without any interference to the biological system, there is considerable interest in their utilization to label and track small molecules on live cells.\(^1\) Commonly used bioorthogonal reactions that meet these criteria are Staudinger ligations,\(^2\) copper(I)-catalyzed azide–alkyne cycloaddition,\(^3\) and strain-promoted azide–alkyne cycloaddition.\(^4\) However, the derivatives of these reactions have poor water solubility or difficulty in synthesizing large quantities. Indeed, improving the reaction kinetics and the biocompatibility of the current reactions needs to be further studied, and the development of new bioorthogonal reactions with high reactivity is urgent. Currently, some reports demonstrated that tetrazines can react rapidly and specifically with strained alkenes to form stable adducts in inverse electron demand Diels–Alder reactions.\(^5\)–\(^7\) This chemistry is orders of magnitude faster than the classical cycloadditions and has been used in live cell labeling.\(^8\)–\(^9\)

Very recently, with the development of IED-DA cycloaddition reaction in bioorthogonal field, the method for specific labeling of proteins in complex biological systems is the currently attractive area. The most popular technique in the modification of proteins is based on the introduction of genetic unnatural amino acids by using bioorthogonal tRNA/tRNA-synthetase pairs.\(^10\),\(^11\) Inspired by recent advances in IED-DA cycloaddition reactions, the repertoire of genetically encoded chemical reactive amino acids grew considerably.\(^12\) And a set of new dienophilic amino acids were synthesized and incorporated into proteins in *E. coli* and mammalian cells through suppressing the amber stop codon.\(^13\)–\(^15\)

Many of the synthetic unnatural amino acids were connected by a ligand or a linker between these functional tags and natural amino acid groups.\(^16\)–\(^18\) This method provides a easy way to synthesis a wide range of unnatural amino acids with diverse side chains, but this kind amino acids also have many deficiencies, such as unstability, difficult to handle, and complex structure may affecting the function. While the *de novo* synthesis of unnatural amino acids provides another way to overcome this defects, and many method had developed.\(^19\)–\(^21\) But the *de novo* synthesis of unnatural amino acids for IED-DA reactions was almost no literature reports. The toolbox of unnatural amino acids was further expanded by our recent report, in which tetrazine group was connected directly to the benzene ring of phenylalanine and a tetrazine-containing amino acid was synthesized.\(^22\) The tetrazine amino acid has shown to be stable enough to be used for peptide modification and live cell labeling.

In this study, based on the known diversity of hydrophilic dienes, we sought to *de novo* synthesize an unnatural amino acid containing a hydrophilic diene group. As a reaction partner for the tetrazines, bicyclo[6.1.0]nonyne (BCN) group was selected as a model dienophile substrate for the unnatural amino acid synthesis, which possessed a higher reactivity than many hydrophilic dienes because of its enhanced cyclopropane fusion reactivity.\(^23\) Moreover, unlike other strained alkenes, BCN reacted with tetrazine to give a single product of defined stereochemistry. Besides, the compound was easily obtained in a highly straightforward process through cyclopropanation of 1,5-cyclooctadiene.
Results and discussion

Synthesis and characterization of L-BCN-containing amino acid

Just as the literature reported, compound 6 can be synthesized in four steps started by the dropwise addition of ethyl diazoacetate to excess 1,5-cyclooctadiene in the presence of rhodium acetate, to give a mixture of diastereomeric compounds exo-3 and endo-3. Next, the individual stereoisomer exo-3 was selected because of its higher yield, and was converted into the corresponding hydroxalkyne product by the reduction of the ester group, bromination, and elimination, to give the compound BCN group.

Compound 11 can be synthesized in five steps started by the oxidation of alcohol of BCN to the aldehyde under Swern conditions, followed by the Strecker reaction to nitrile. One key step in the reaction was the hydrolysis of the intermediate nitrile 8 with 2 M NaOH to afford BCN amino acid. While by contrast, in experimenting we found that the hydrolysis of the intermediate nitrile 8 with 6 M HCl and 10% aqueous H2SO4 led to an entirely conversion of 8 into by-product ketone amino acid. Attempts of enantiomeric resolution of 8 with recombinate nitrilase from Arabidopsis thaliana (EC 3.5.5.1) failed. At last, the amino acid 9 was acetylated with Ac2O and the resulting Ac-BCN was enantiomericly resolved with kidney acylase l to produce L-BCN amino acid (Scheme 1).

To test whether the configuration of the product was single, the Nz-(2,4-dinitro-5-fluorophenyl)-l-alanine amide (FDAA) reagent was selected for chiral analysis, and the results confirmed the enantiomeric purity of the compound. Compound 11 was sufficiently stable for prolonged storage at 20 °C and did not undergo any structural changes upon stirring in the presence of PBS solution.

Synthesis and characterization of tetrazine-containing amino acid

Next, we aimed to synthesize tetrazine-containing amino acids (Scheme 2). We all knew that 1,2,4,5-tetrazines were pretty reactive toward water, which made them unsuitable for biocoujugation. While, the stability of tetrazines can be dramatically improved by the substitution with aromatic groups. Our previously synthesized tetrazine amino acid 13 showed excellent stability in PBS and biological media, with little or no decomposition after prolonged exposure at room temperature, but was unstable in 20% piperidine/DMF. In general, tetrazines, which was substituted with electron donating groups like alkyl, tended to be more stable with slower cycloaddition kinetics. According to the above summarized theory, we added a methyl in tetrazine of compound 13 to give another tetrazine amino acid (S)-2-amino-3-(4-(6-methyl-1,2,4,5-tetrazin-3-yl) phenyl) propanoic acid 14, which demonstrated a good balance of solution stability and fast reaction kinetics.

The tetrazine amino acid 14 was synthesized in a way similar to compound 13. Compound 14 was prepared starting from the commercially available 4-cyano-L-phenylalanine with acetaldehyde hydrochloride and anhydrous hydrazine in the presence of elemental sulfur. The initial product dihydrotetrazine derivative was oxidized to the tetrazine by treating with sodium peroxide in acetic acid. Moreover, compound 14 showed excellent stability in 20% piperidine/DMF with little or no decomposition observed after prolonged exposure at room temperature (see Fig. S2†), a prerequisite for peptide synthesis via an Fmoc synthetic strategy.

Kinetic experiments between BCN-containing amino acid and tetrazine-containing amino acids

We have synthesized a BCN-containing amino acid and tetrazine-containing amino acids. Alternatively, we sought to directly select the reaction of the BCN amino acid and tetracine amino acids as bioorthogonal chemical “handles” for amino acid coupling via the IED-DA reactions. The rate constants of the reactions between BCN amino acid and tetracines, a prerequisite in vivo applications, was determined by manual
mixing under the pseudo-first-order conditions. By following the exponential decay of the tetrazine absorbance at 523 nm upon reaction with a 10–100 fold excess of BCN, we determined the rate constants of the reactions between BCN amino acid and tetrazine 13 as \( k_1 = (437 \pm 13) \text{M}^{-1} \text{s}^{-1} \) (see Fig. S3†). Under the same conditions, the rate constants of the reactions between BCN amino acid and tetrazine 14 was determined with UV/vis spectroscopy following the decay in the absorption of tetrazine derivative at 527 nm. The results showed that \( k_2 = (1.45 \pm 0.05) \text{M}^{-1} \text{s}^{-1} \) (see Fig. S4†). Obviously, the reactions between two tetrazine amino acids and BCN amino acid were so reactive and suitable for rapid biological labeling.

1-BCN-containing amino acid in the epidermoid carcinoma cells labeling

Specific labeling of living cell was one of the most valuable applications in bioorthogonal chemistry.41,42 To prove the possible biological application of the 1-BCN amino acid, we chose to label epidermal growth factor receptors (EGFR), which played an important role in cancer-cell signaling and was considered as the key target for therapeutic inhibition, with the anti-EGFR monoclonal antibody cetuximab. Commercially available cetuximab was marked with tetrazine amino acid of using IED-DA reaction was suitable for rapid biological labeling.

Fig. 1 Compound 11 reacted with Boc-protected tetrazine amino acid to label cancer cells. Cancer cells A431, which overexpressed EGFR, were exposed to the Cetuximab antibodies modified with tetrazine and 5-carboxyfluorescein (green). In the next step, the pretargeted cells were labeled with a BCN bearing a fluorophore such as Cy5.5 (red).

In conclusion, we reported a general method for de novo synthesis of a BCN group containing amino acid, and used

Experimental

All the experimental procedures and supporting figures are reported in the ESI†

Conclusions

In conclusion, we reported a general method for de novo synthesis of a BCN group containing amino acid, and used...
Marfey’s reagent for chiral analysis, which laid the foundation for being introduced into peptides based on the solid-phase synthesis method. Moreover, as a reaction partner for the BCN amino acid, we also de novo synthesized another tetrazine containing amino acid 14 in a way similar to compound 13, which was synthesized in our previous work. We demonstrated that the reactions of BCN amino acid and tetrazine amino acids can be used as a bioorthogonal chemical “handle” for amino acid coupling via the IED-DA reaction. Finally, the high reaction rate of BCN amino acid and tetrazine amino acids was suitable for cancer cell labeling under physiological conditions. Our future efforts will focus on the incorporation of this BCN amino acid site-directly in peptide modification.

Conflicts of interest
There are no conflicts to declare.

Acknowledgements
The authors want to thank Prof. Lingyun Mu for the Fluorescent microscope experiments.

References
3. V. V. Rostovtsev, L. G. Green, V. V. Fokin and K. B. Sharpless, Angew. Chem., 2002, 114, 2708-2711.