Organic & Biomolecular Chemistry

Dynamic Article Links

Cite this: *Org. Biomol. Chem.*, 2011, **9**, 7303

www.rsc.org/obc

COMMUNICATION

Exploring isonitrile-based click chemistry for ligation with biomolecules†

Henning Stöckmann,[a,b] André A. Neves,[b] Shaun Stairs,[a] Kevin M. Brindle[b] and Finian J. Leeper*[a]

Received 19th August 2011, Accepted 24th August 2011
DOI: 10.1039/c1ob06424j

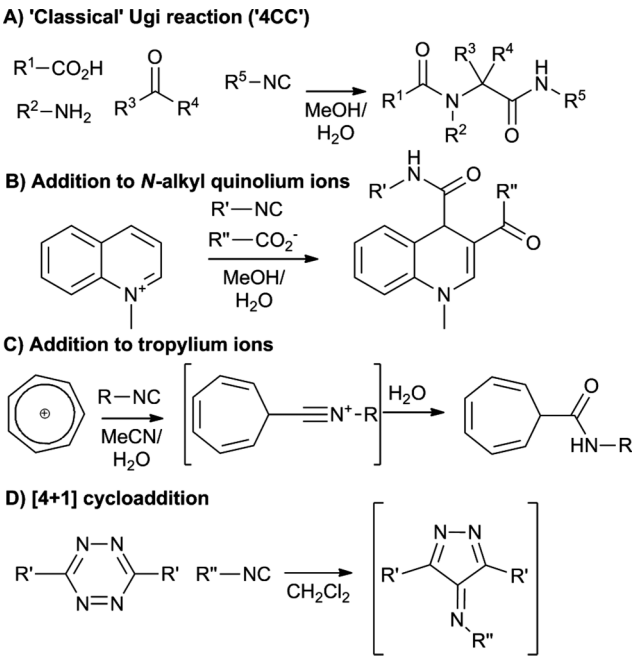
We show here that isonitriles can perform click reactions with tetrazines in aqueous media, making them promising candidates for ligation reactions in chemical biology and polymer chemistry. This is the first time that a [4+1] cycloaddition has been used as a biocompatible ligation reaction.

Ligation reactions are of great importance in areas such as modification of biomolecules, supramolecular chemistry, material science and nanotechnology.[1] Examples of bioorthogonal ligation reactions are (a) the condensation of ketones and aldehydes with amine nucleophiles such as hydrazines and hydroxylamines, (b) the Staudinger ligation of azides and phosphines and the [3 + 2] cycloaddition of azides and alkynes[2] and (c) the inverse electron demand Diels–Alder reaction between tetrazines and *trans*-cyclooctenes.[3] The relatively small number of bioorthogonal ligation reactions reflects the pressing need for the exploration of new orthogonal chemical reporter groups.

Here we investigate the possibility of using the isonitrile group for ligation reactions. The isonitrile group is a two-atom functional group that has been known since 1867[4] and it is one of the few examples of a stable chemical compound with a divalent carbon. Its rate of hydrolysis into the corresponding formamide is negligible around neutral pH values.[5] Its small size may have distinct advantages for labelling

$$[R–N{=}C: \leftrightarrow R–N^{+} \equiv C^{-}]$$

biomolecules, due to the minimal perturbation of the biological system being studied. Isonitriles were monitored toxicologically by Bayer AG in the 1960's and shown to exhibit no appreciable toxicity for mammals, with oral and subcutaneous doses of 500–5000 mg kg^{-1} being tolerated by rodents.[6] Like the widely used azido group, the isonitrile group is not found in mammals. However, numerous isonitrile-bearing natural products have been found in marine and terrestrial sources.[7] Isonitriles are weak nucleophiles lacking reactivity towards simple ketones, aldehydes or imines. Suitable reaction partners for isonitriles are resonance-stabilized electrophilic carbocations. Iminium ions,[8] *N*-alkylquinolinium ions[9] and tropylium ions[10] all react with isonitriles by α-addition, which is usually followed by a secondary reaction with a suitable anion (Scheme 1). In addition, activated carbonyl compounds such as 1,1,1,5,5,5- hexafluoropentane-2,4-dione can add isonitriles in a 1 : 1 manner.[11] Isonitriles can also react with carboxylates at elevated temperatures and function as ligands for a number of transition metals.[12]

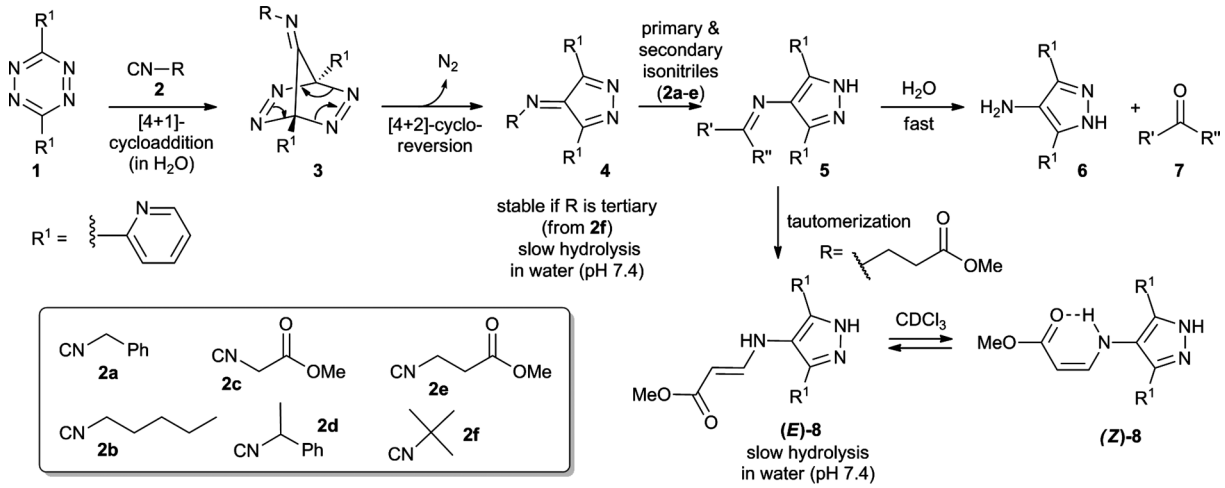


Scheme 1 Candidate reactions with isonitriles.

Ugi reactions are water-compatible[13] and a few reports exist where Ugi reactions have been used to label biomolecules under physiological conditions.[5,14] A bifunctional azide-isonitrile conjugating agent combining [3 + 2] cycloaddition click chemistry and Ugi chemistry has recently been reported.[15] However, biomolecular functional groups such as amines and carboxylic acids, which are abundant on proteins, could interfere with Ugi-type reactions and thus decrease their efficiency. Instead we investigated a [4 + 1]-cycloaddition between a selection of isonitriles and 3,6-di-2-pyridyl-1,2,4,5-tetrazine (**1**, R_1 = 2-pyridyl) (Scheme 2). This [4 + 1]-cycloaddition has only been reported in two papers by Seitz and co-workers, who used it to prepare pharmacologically interesting aminopyrazoles from benzyl isonitrile **2a** and a range of substituted tetrazines (**1**). The first step of the reaction sequence is the formation of non-isolable tetraazanorbornadienimine

[a]*University of Cambridge, Department of Chemistry, Lensfield Road, Cambridge, UK, CB2 1EW. E-mail: fjl1@cam.ac.uk; Fax: +44 (0) 1223 336 362; Tel: +44 (0) 1223 336 403*
[b]*Cancer Research UK Cambridge Research Institute, Li Ka Shing Centre, Robinson Way, Cambridge, UK, CB2 0RE*

† Electronic supplementary information (ESI) available: Experimental data and NMR spectra. See DOI: 10.1039/c1ob06424j



Scheme 2 The [4 + 1] cycloaddition between isonitriles and tetrazines **1**.

derivatives **3** which spontaneously undergo a [4 + 2]-cycloreversion with release of N_2 to result in 4*H*-pyrazol-4-imine derivatives **4**. These intermediates could not be isolated as they tautomerized to the aromatic pyrazoles **5**, which then hydrolysed to give the desired aminopyrazoles **6**.[16]

Because of the hydrolytic lability of imine **5**, this reaction cannot be used in its reported form for chemical ligation reactions in water. Thus, we investigated the possibility of modulating the course of the reaction by modifying the structure of the isonitriles being used and thus turning it into an effective aqueous ligation reaction. We also investigated water-compatibility and reaction rates, neither of which have been reported in the literature.

When isonitriles **2a** to **2d** were used in the reaction sequence, the 4*H*-pyrazol-4-imine could not be isolated. Instead, rapid tautomerisation to imines **5** occurred, followed by spontaneous hydrolysis to aminopyrazole **6** by traces of moisture. For the case of the simple alkyl isonitrile **2b** the reaction was followed by NMR spectroscopy in $CDCl_3$ (Figure S2, ESI†). The intermediate imine **5b** was the main component after 10 min but some hydrolysis by traces of water in the solvent had already occurred and aldehyde and aminopyrazole were major components after 24 h. In MeOH–H_2O (1 : 1) hydrolysis to give the aldehyde is rapid. Secondary isonitrile **2d** was converted into the corresponding ketone **7d** in the same manner. Although the fast hydrolysis limits the reaction's usefulness for conjugation reactions, it does provide a novel way to mask carbonyl groups as isonitriles and unmask them with tetrazines, effectively providing a new protecting group strategy for aldehydes and ketones.

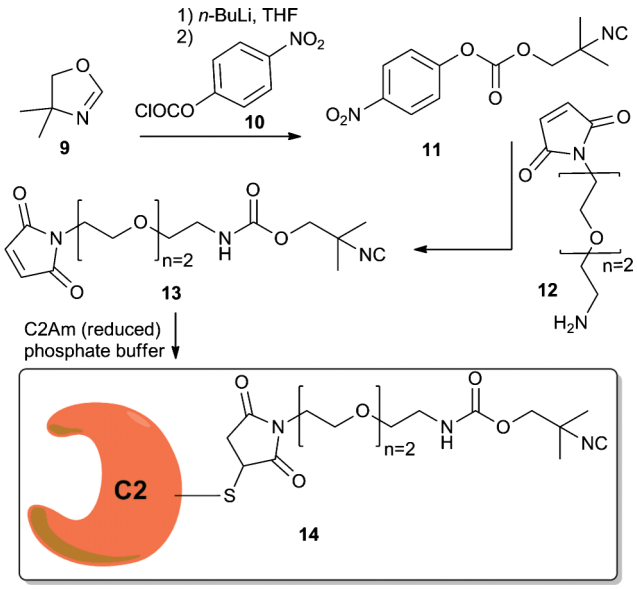
In the case of isonitrile **2e**, imine **5e** did not hydrolyse (even when the reaction was performed in THF–H_2O, 1 : 1), but instead tautomerized again into the stable α,β-unsaturated system **8** (a vinylogous urethane). Although the reaction predominantly yielded the *trans*-olefin, this slowly isomerised into the *cis*-olefin in $CDCl_3$ due to the intramolecular hydrogen bond.[17] In D_2O–CD_3CN the proton adjacent to the ester exchanges for deuterium, showing that enamine–imine tautomerisation does occur. The reaction rate for the [4 + 1]-cycloaddition was determined by UV–vis spectroscopy in MeOH at 25 °C to be (5.2 ± 0.9) × 10^{-2} M^{-1} s^{-1} for isonitrile **2e**. This rate is comparable to that for the [3 + 2]-cycloaddition reaction of strained cyclooctynes, such as difluorocyclooctyne (DIFO), with benzyl azide, (4.2 ± 0.1) × 10^{-2} M^{-1} s^{-1} in CD_3CN.[18] Adduct **8** showed reasonable stability in buffered aqueous systems with a half-life of *ca.* 16 h in phosphate buffered saline (PBS, pH 7.4)–CD_3CN (1 : 1) as determined by ^{1}H-NMR spectroscopy.

Table 1 Rate constants for [4 + 1] cycloadditions and half-lives of adducts in PBS–CD_3CN (1 : 1)

Isonitrile	*k* (MeOH) (× 10^{-2} M^{-1} s^{-1})	*k* (THF–H_2O (1 : 1)) (× 10^{-2} M^{-1} s^{-1})	$t_{1/2}$ (h)
2e	5.2 ± 0.9	12.4 ± 0.7	16
2f	7.7 ± 0.3	57.5 ± 1.5	63

Next *tert*-isonitrile **2f** was tested as the imine **4f** cannot tautomerize. The reaction rate for the [4 + 1]-cycloaddition in MeOH at 25 °C was determined to be (7.7 ± 0.3) × 10^{-2} M^{-1} s^{-1}, slightly higher than that of primary isonitrile **2e**. In H_2O–THF (1 : 1), a 7-fold rate-enhancement was found (Table 1). Despite its electron-deficient character, imine **4f** was found to be surprisingly stable in aqueous systems with a half-life of *ca.* 63 h in PBS–CD_3CN (1 : 1). Having shown that both tertiary isonitriles and isocyanopropionic acid-based isonitriles are, in principle, suitable for ligation reactions with tetrazine **1** in aqueous systems, the [4 + 1]-cycloaddition was next tested in a protein system to show that the chemistry is biocompatible.

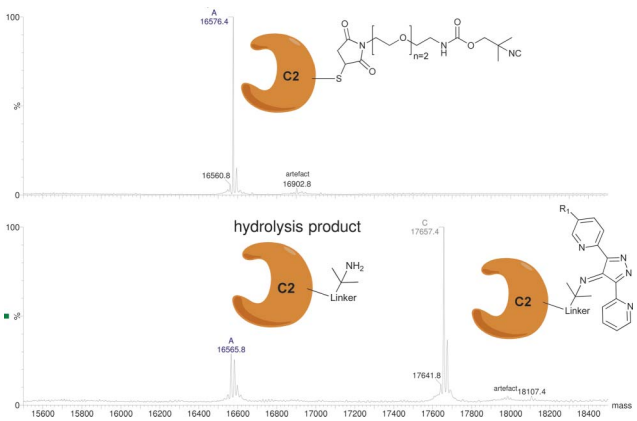
A convenient method to prepare a cysteine-reactive isonitrile linker started with 4,4-dimethyl-2-oxazoline **9** (Scheme 3), which was deprotonated with *n*-BuLi and readily fragmented into the isonitrile-alkoxide.[19] This alkoxide was reacted with *para*-nitrophenylchloroformate **10** to give the corresponding carbonate **11**, which is stable indefinitely at room temperature and readily forms carbamates upon reaction with primary amines such as **12**. The resulting hetero-bifunctional linker **13** was reacted with a mutant of the C2A domain of synaptotagmin-I having a single cysteine residue at position 78[20] (C2Am, MW = 16222 Da) *via* conjugate addition of its cysteine to the maleimide unit of **13** (full conversion, Scheme 3). The identity of C2Am–isonitrile adduct **14** was confirmed by mass spectrometry (MW = 16576 Da, Scheme 4) and no byproducts were detected.



Scheme 3 Synthesis of an isonitrile linker and attachment to the protein C2.

Although potassium 3-isocyanopropanoate and its esters and amides are stable compounds, maleimides were found to be incompatible with primary isonitriles. Thus, similar conjugation of **2e** to C2Am has not yet been successful.

The C2Am–*tert*-isonitrile (60 μM) was incubated with a tetrazine–rhodamine (540 μM, 18 h, see ESI† for details). This resulted in the formation of the 4*H*-pyrazol-4-imine adduct (MW = 17657 Da, full conversion), accompanied by a small amount of its *tert*-amine hydrolysis product (MW = 16566 Da, Scheme 4).



Scheme 4 Conjugation of C2A–isonitrile (top) with tetrazine–rhodamine (bottom). R_1 = rhodamine (see ESI† for structural details).

In summary, a biocompatible conjugation reaction of isonitriles has been investigated. The tetrazine-reagent **1** underwent [4 + 1] cycloadditions with primary, secondary and tertiary isonitriles in aqueous solution. The final outcome of the reaction depended on the substituents on the isonitrile. With ordinary primary and secondary isonitriles tautomerization and hydrolysis converts the isonitrile into a carbonyl group, but isocyanopropanoates and tertiary isonitriles form adducts with tetrazines that only hydrolyse slowly in buffered aqueous systems. This chemistry has been shown to be biocompatible by using it for fluorophore conjugation to a tertiary isonitrile-tagged protein. This is the first time that a [4 + 1] cycloaddition has been used as a biocompatible ligation reaction. It could potentially be used for the ligation of other biomolecules, such as sugars, nucleic acids or natural products, in addition to proteins.

We wish to thank Cancer Research UK for funding this work and Dr Len Packman, Protein and Nucleic Acid Chemistry Facility, Department of Biochemistry, Cambridge, UK, for expert support in MS analysis.

References

1 (*a*) H. C. Kolb, M. G. Finn and K. B. Sharpless, *Angew. Chem., Int. Ed.*, 2001, **40**, 2004–2021; (*b*) E. M. Sletten and C. R. Bertozzi, *Angew. Chem., Int. Ed.*, 2009, **48**, 6974–6998; (*c*) J. E. Moses and A. D. Moorhouse, *Chem. Soc. Rev.*, 2007, **36**, 1249–1262.
2 (*a*) J. C. Jewett and C. R. Bertozzi, *Chem. Soc. Rev.*, 2010, **39**, 1272–1279; (*b*) W. H. Binder and R. Sachsenhofer, *Macromol. Rapid Commun.*, 2008, **29**, 952–981; (*c*) H. Stöckmann, A. A. Neves, S. Stairs, H. Ireland-Zecchini, K. M. Brindle and F. J. Leeper, *Chem. Sci.*, 2011, **2**, 932–936.
3 (*a*) F. Thalhammer, U. Wallfahrer and J. Sauer, *Tetrahedron Lett.*, 1990, **31**, 6851–6854; (*b*) M. L. Blackman, M. Royzen and J. M. Fox, *J. Am. Chem. Soc.*, 2008, **130**, 13518–13519; (*c*) H. Stöckmann, A. A. Neves, H. A. Day, S. Stairs, K. M. Brindle and F. J. Leeper, *Chem. Commun.*, 2011, **47**, 7203–7205.
4 (*a*) A. W. Hofmann, *Liebigs Ann. Chem.*, 1867, **144**, 114; (*b*) A. Gautier, *Liebigs Ann. Chem.*, 1867, **142**, 289.
5 L. Goldstein and A. Niv, *Appl. Biochem. Biotechnol.*, 1993, **42**, 19–35.
6 (*a*) I. Ugi, U. Fetzer, U. Eholzer, H. Knupfer and K. Offermann, *Angew. Chem., Int. Ed. Engl.*, 1965, **4**, 472–484; (*b*) I. Ugi, *Isonitrile Chemistry*, Academic Press, New York, 1971, pp. 1–278.
7 (*a*) M. J. Garson and J. S. Simpson, *Nat. Prod. Rep.*, 2004, **21**, 164–179; (*b*) N. Fusetani, *Nat. Prod. Rep.*, 2004, **21**, 94–104.
8 (*a*) I. Ugi, *Angew. Chem., Int. Ed. Engl.*, 1962, **1**, 8; (*b*) I. Ugi, *Angew. Chem.*, 1962, **74**, 9.
9 I. Ugi and U. Böttner, *Liebigs Ann. Chem.*, 1963, **670**, 74.
10 I. Ugi, W. Betz and K. Offermann, *Chem. Ber.*, 1964, **97**, 3008–3011.
11 A. Shaabania, A. Bazgira, K. Soleimania and H. R. Bijanzahdeh, *J. Fluorine Chem.*, 2002, **116**, 93–95.
12 (*a*) J. A. Weigel, K. K. P. Srivastava, E. P. Day, E. Munck and R. H. Holm, *J. Am. Chem. Soc.*, 1990, **112**, 8015–8023; (*b*) T. G. Traylor and D. V. Stynes, *J. Am. Chem. Soc.*, 1980, **102**, 5938–5939.
13 M. C. Pirrung and K. D. Sarma, *J. Am. Chem. Soc.*, 2004, **126**, 444–445.
14 (*a*) A. Freeman, M. Soklovsky and L. Goldstein, *Biochim. Biophys. Acta*, 1979, **571**, 127–136; (*b*) T. Ziegler, S. Gerling and M. Lang, *Angew. Chem., Int. Ed.*, 2000, **39**, 2109–2112.
15 V. G. Nenajdenko, A. V. Gulevich, N. V. Sokolova, A. V. Mironov and E. S. Balenkova, *Eur. J. Org. Chem.*, 2010, **2010**, 1445–1449.
16 (*a*) P. Imming, R. Mohr, E. Müller, W. Overheu and G. Seitz, *Angew. Chem., Int. Ed. Engl.*, 1982, **21**, 284; (*b*) P. Imming, R. Mohr, E. Müller, W. Overheu and G. Seitz, *Angew. Chem.*, 1982, **94**, 291; (*c*) R. J. Xiao-Guang Yang and G. Seitz, *Arch. Pharm.*, 1991, **324**, 923–925.
17 J. M. Lee, D.-S. Ahn, D. Y. Jung, J. Lee, Y. Do, S. K. Kim and S. Chang, *J. Am. Chem. Soc.*, 2006, **128**, 12954–12962.
18 J. A. Codelli, J. M. Baskin, N. J. Agard and C. R. Bertozzi, *J. Am. Chem. Soc.*, 2008, **130**, 11486–11493.
19 T. Lindhorst, H. Bock and I. Ugi, *Tetrahedron*, 1999, **55**, 7411–7420.
20 I. S. Alam, A. A. Neves, T. H. Witney, J. Boren and K. M. Brindle, *Bioconjug. Chem.*, 2010, **21**, 884–891.