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Exploring Condensation Reaction Between Aromatic Nitriles and Amino Thiols to Form Nanoparticles in Cells for Imaging the Activity of Protease and Glycosidase

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Abstract

The condensation reaction between 6-hydroxy-2-cyanobenzothiazole (CBT) and cysteine has been shown for various applications such as site-specific protein labelling and in vivo cancer imaging. This report further expands the substrate scope of this reaction by varying the substituents on aromatic nitriles and amino thiols and testing their reactivity and ability to form nanoparticles for

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cell imaging. The structure-activity relationship study leads to the identification of the minimum structural requirement for the macrocyclization and assembly process in forming nanoparticles. One of the scaffolds made of 2-pyrimidinecarbonitrile and cysteine joined by a benzyl linker was applied to design fluorescent probes to image caspase-3/7 and β -galactosidase activity in live cells. These results demonstrate the generality of this system for imaging hydrolytic enzymes.

Graphical Abstract

Tuning condensation reaction for target-activated self-assembly of nanoparticles in cells:

Scope of condensation reaction between aminothiols and cysteine and structure of the molecular scaffold are investigated for target-activated cyclization and self-assembly of nanoparticles in cells. The optimized scaffold was used to generate enhanced signal retention when imaging caspases and β -galactosidase activities in cells.

Keywords

self-assembly; bioorthogonal reactions; nanoparticles; enzyme activity imaging

Introduction

Bioorthogonal reactions have become important tools to probe biological processes, design new functions and forge novel interactions.^[1] Well known examples include ketone/hydroxylamine condensation,^[2] Staudinger ligation,^[3] strain-promoted azide-alkyne cycloaddition,^[4] photo-click cycloaddition^[5] and Diels-Alder cycloaddition between tetrazines and strained alkenes/alkynes.^[6] These reactions have been applied to site-directed protein labeling,^[1] studying metabolic pathways and engineering biomaterials.^[3b, 3d, 4b, 8] The applicability of these reactions to live systems is however often compromised by issues like insufficient stability or suboptimal reaction rates.^[1, 4a-c, 9] For example, some of the most reactive tetrazines have been found to be prone to hydrolysis and intracellular cysteine attack.^[9] The use of large hydrophobic moieties such as cyclooctyne to improve the reactivity also increases the potential for nonspecific binding to membrane structures.^[1] Recently, efforts have been made to modulate the reactivity of bioorthogonal reactions by light.^[10] Very few reactions reported to date can be activated by an enzyme. For example, Fox and coworkers reported horseradish peroxidase activated oxidation of dihydrotetrazine to tetrazine^[11] and Laughlin and coworkers reported enzymatic uncaging of a steric

blocking group on cyclopropene.^[12] However, no enzyme activated bioorthogonal reactions have been used *in vivo*.

We have been interested in the reaction between 6-hydroxy-2-cyanobenzothiazole (CBT) and D-cysteine that originates from the synthesis of firefly D-luciferin (Scheme 1).^[13] This reaction can proceed under physiological conditions with a high second-order rate constant ($9.19 \text{ M}^{-1} \text{ s}^{-1}$), which is 120 times faster than that of a typical copper-free azide-alkyne cycloaddition ($7.6 \times 10^{-2} \text{ M}^{-1} \text{ s}^{-1}$).^[4b] Both the amine and thiol groups in the 1,2-aminothiol moieties are required to form the five-membered thiazoline ring. We first demonstrated the site-specific labeling of proteins at terminal cysteine using analogues of 2-cyanobenzothiazole, and others have also used the CBT chemistry for protein labeling.^[14]

An interesting feature of this reaction different from most activatable bioorthogonal reactions is that its reactivity can be modulated by pH, redox potential, or enzyme activity. For example, a disulfide or a peptidyl caging unit can be introduced to mask the reactivity of the thiol or amino groups. Subsequent unmasking by a protease or a reducing agent will restore the reactivity and initiate the condensation reaction. This modular feature has empowered this reaction for various applications such as target-activated molecular assembly in living cells.^[13] A molecular probe can be generated by linking the masked cysteine residue to the CBT unit. Upon unmasking of the amino and thiol groups on the cysteine residue, the intermediate would react with each other in a head-to-tail fashion to form a mixture of cyclic dimers and oligomers that can further interact through hydrophobic and π - π interactions to form nano-aggregates (Scheme 1). It has been demonstrated that pH, reduction, and enzyme-controlled mask cleavage can restore the reactivity of the reaction and trigger the condensation and assembly process *in vitro* and in living cells.^[13, 15] The high reactivity of CBT makes it possible to react with free cysteine, which exists in cells and plasma at various concentrations (20-200 μM).^[16] In order to minimize this side reaction *in vivo*, we replaced CBT with 2-cyano-6-hydroxyquinoline (CHQ) that reacts with free cysteine at a much slower reaction rate ($0.019 \text{ M}^{-1} \text{ s}^{-1}$), so the condensation proceeds preferentially in an intramolecular manner in cells since the intramolecular molarity is much higher than the free cysteine concentration.^[7] The cyclization product will then aggregate to increase retention at target sites to generate imaging contrast (Scheme 1). This intramolecular condensation and macrocyclization has been demonstrated in imaging caspases activity *in vivo* with fluorescence imaging,^[17] positron emission tomography (PET),^[18] and magnetic resonance imaging (MRI).^[19]

In order to further understand how general this macrocyclization reaction and assembly process is and whether it can occur to structures other than CBT, we investigated the scope of this condensation reaction and the minimum structural requirement for the macrocyclization and assembly process. We first optimized the aromatic nitrile and aminothiol units by modulating their electronic and steric properties. The linking group between the two units also plays an important role in promoting nano-assembly through hydrophobic and π - π interactions. We then investigated both the effects of aromaticity and length of the linker group on the macrocyclization and nano-assembly process. Renewed understanding of this condensation reaction allowed us to design a new molecular scaffold. We then applied this new platform for imaging two different types of hydrolytic enzymes in

living cells: caspases-3, a peptidase and β -galactosidase, a glycoside hydrolase, and demonstrated the general applicability of this condensation reaction to enable the target-enabled in situ ligand assembly for imaging the activity of enzymes.

Results and Discussion

Analysis and Rational Design of Bioorthogonal Condensation Substrate Pairs.

Based on the known examples of condensation reaction between aromatic nitriles and aminothiols, we postulated that the reactivity of the aromatic nitrile was affected by π electron density and number of heteroatoms in the aromatic ring. For example, for CBT, CHQ and 2-pyridinecarbonitrile (entries 1 - 3 in Table 1), no reaction was observed for 2-pyridinecarbonitrile while modest to fast reaction rates were observed for CHQ ($0.06 \text{ M}^{-1} \text{ s}^{-1}$) and CBT ($2.9 \text{ M}^{-1} \text{ s}^{-1}$). Comparison between the structures of CHQ and 2-pyridinecarbonitrile (entries 2 and 3 in Table 1) reveals that adding one more aromatic ring in CHQ significantly increases the reactivity of aromatic nitrile by providing better electron delocalization in the fused aromatic ring system. Compare CBT with CHQ (entries 1 and 2 in Table 1), the presence of different heteroatoms like nitrogen or sulfur in the aromatic ring significantly changes the reactivity of the aromatic nitriles since heteroatoms have different electronegativity and number of unshared electron pairs from carbon. These observations prompted us to test if the reactivity of aromatic nitriles for the condensation reactions could be modulated by tuning the electronic properties of the heteroatoms on the aromatic ring. We started from the un-reactive 2-pyridinecarbonitrile (entry 3 in Table 1) to design reactive aromatic nitriles by adding more nitrogen atoms onto the aromatic ring especially at 3 or 5-position and/or by adding electron-withdrawing groups such as halide. We first tested 2-pyrimidine-carbonitrile and 5-bromo-2-pyrimidinecarbonitrile and measured their 2nd order rate constants with L-cysteine using an HPLC assay (entry 5 and 4 in Table 1): $1.0 \text{ M}^{-1} \text{ s}^{-1}$ for 2-pyrimidinecarbonitrile and $2.21 \text{ M}^{-1} \text{ s}^{-1}$ for 5-bromo-2-pyrimidinecarbonitrile. We also synthesized an alkoxy-substituted 2-pyrimidinecarbonitrile (entry 6 in Table 1) and tested its reactivity with L-cysteine. Since alkoxy substituents are electron donating, its reactivity ($0.01 \text{ M}^{-1} \text{ s}^{-1}$) is much lower than 2-pyrimidinecarbonitrile.

The nucleophilicity of the aminothiol is another determining factor of the reaction rate and a more reactive aminothiol may provide better selectivity over intermolecular reaction with endogenous free cysteine. Previously we compared the reactivity of a series of aminothiols with different electronic and conformational properties and found L-cysteine to be the best among all that were tested.^[7] Here we examined more derivatives of cysteine. We first determined the 2nd order rate constant of the condensation between D-penicillamine (3,3-dimethyl D-cysteine) and CBT (entry 7 in Table 1) to be $0.029 \text{ M}^{-1} \text{ s}^{-1}$, which was dramatically lowered than that of L-cysteine ($2.9 \text{ M}^{-1} \text{ s}^{-1}$). In spite of the “gem-dimethyl effect” on cysteine,^[20] steric hindrance introduced by the two methyl groups around the thiol nucleophile lowers the rate of intermolecular nucleophilic attack. Interestingly, 2-methyl-L-cysteine showed a higher reaction rate constant ($5.4 \text{ M}^{-1} \text{ s}^{-1}$, entry 8 in Table 1) than that of L-cysteine ($2.9 \text{ M}^{-1} \text{ s}^{-1}$).

Structural Requirements for Linker to Promote Self-assembly.

We have shown that a replacement of the luciferin moiety in the previously reported scaffold by a non-aromatic, flexible PEG linker failed to produce nano-aggregates after condensation.^[17] This observation indicates the key role of the luciferin moiety in promoting self-assembly, however, it was unclear if other rigid aromatic structures besides the luciferin moiety could similarly promote assembly of the cyclization product. To test this hypothesis, we designed compounds I and II, both of which use a benzyl group as the linker in place of the luciferin moiety (Figure 1a). Compound II differs from compound I in that there is one more amino acid residue in the linker so that the formed macrocyclic ring would have a similar size to that having the luciferin moiety. Upon adjustment of the pH value from 4 to 7.4, both I and II afforded cyclized products, as confirmed by HPLC and mass spectrometry. Their first-order reaction rate constants were determined by an HPLC assay: $0.2 \times 10^{-3} \text{ s}^{-1}$ for compound I and $1.2 \times 10^{-3} \text{ s}^{-1}$ for compound II; and dynamic light scattering (DLS) analysis of their cyclized products (cycl-I and cycl-II, Figure 1b) showed formation of nano-aggregates with hydrodynamic sizes of around 80 nm for both compounds. These results suggest that like the luciferin moiety, aromatic functional groups as simple as a benzene ring can similarly promote assembly of cyclic products.

To test if the benzyl linker can promote macrocyclization and self-assembly of aromatic nitriles other than CBT and CHQ, we designed compounds III, IV, V, and VI, each of which has the luciferin-linker replaced by a benzyl linker, and CHQ by an alkoxy-substituted 2-pyrimidinecarbonitrile. As shown in Figure 1, despite different cyclization kinetics and condensation substrate pairs, all their cyclic products were able to form nano-aggregates ranging from 80 nm to 250 nm in diameters, demonstrating the robustness of this simple aromatic linker in promoting self-assembly of cyclic products. Length of the linker is also important to the cyclization kinetics. From compound III and IV to V, the kinetics of IV ($2.7 \times 10^{-3} \text{ s}^{-1}$) is the fastest while the ring size of its macrocyclic product is between III ($0.9 \times 10^{-3} \text{ s}^{-1}$) and V ($0.8 \times 10^{-3} \text{ s}^{-1}$). When 2-methyl-L-cysteine moiety is introduced to replace cysteine moiety in compound IV, the resulted compound VI displayed a 1.3-fold increase in the cyclization kinetics ($2.7 \times 10^{-3} \text{ s}^{-1}$) over IV ($2.1 \times 10^{-3} \text{ s}^{-1}$), similar to the enhancement in the second order reaction rate constant observed between cysteine moiety and 2-methyl-cysteine moiety in their reaction to CBT (Table 1 entry 1 and 8). It is interesting to observe that despite different kinetics, all of these compounds can undergo macrocyclization and self-assembly. This result suggests that the luciferin moiety in SNAT is not indispensable for macrocyclization and self-assembly. Some aromatic functionalities are necessary, and they can be the luciferin moiety or as simple as a benzyl group.

New Molecular Scaffold for Nanoaggregation.

Among the scaffolds tested in Figure 1a, SNAT4 is of interest for macrocyclization promoted nanoaggregation because of its simplified structure and fast cyclization rate. Based on the condensation reaction rate between alkoxy-substituted 2-pyrimidinecarbonitrile ($0.01 \text{ M}^{-1} \text{ s}^{-1}$) and the intramolecular cyclization rate of compound IV ($2.1 \times 10^{-3} \text{ s}^{-1}$), we estimated the intramolecular effective molarity to be around 210 mM, which is significantly higher than endogenous free cysteine in cells and plasma (usually around 20-100 μM), suggesting the scaffold is unlikely to react with intracellular cysteine. Consistent with this

estimate, in a competitive HPLC assay using this new scaffold (Figure S3), the ratio between the products of intramolecular cyclization and intermolecular condensation is about 45.5:54.5 at 200 mM of free cysteine.

To examine any potential interactions between this new scaffold and intracellular proteins, disulfide caged SNAT4 was incubated in HeLa lysate at 100 μ M at 37 °C for 6 hours. The cell lysates were first labeled with Cy5-azide through click reaction followed by being analyzed by SDS-Page for detection of SNAT4-protein conjugates. We used 6-heptynoic NHS ester to label BSA as the positive control to validate the in-gel labeling condition. BSA was labeled with 6-heptynoic NHS ester, followed by a similar Click reaction with Cy5-azide to provide a positive control. The in-gel fluorescence analysis did not show any significant protein labeling by SNAT4 (Figures 2a) and HPLC analysis of the cell lysate showed the majority of SNAT4 were converted into the cyclized product (Figures 2b and 2c). These results suggest that the lack of reactions of SNAT4 with endogenous proteins. SNAT2 bearing CHQ was also included as a comparison, and consistent with previous report,^[7] the CHQ didn't show any labeling of endogenous molecules either. We further compared them in imaging enzyme activity in apoptotic cells and healthy cells.

Imaging of Glycoside Hydrolase Activity in Live Cells.

We first tested if the new scaffolds could be applied to image β -galactosidase, a widely used gene reporter and biomarker in various diseases and biological processes such as tumor metastasis and cellular senescence.^[21] To image activity of β -galactosidase, a self-immolative group was introduced to link the C-1 hydroxyl group of the galactose to the amino group of the scaffold (Figure 3a). After cleavage of the glycoside by β -galactosidase, the linker self-immolates in the form of quinone methide and carbon dioxide to afford the B-SNAF2/4-BDP-aglycone. Under reducing conditions, the disulfide is reduced followed by intramolecular cyclization between the terminal cysteine and aromatic nitrile and self-assembly into nanoaggregates. To verify this mechanism, B-SNAF2-BDP or B-SNAF4-BDP was incubated with β -galactosidase and TCEP together at 37 °C for 2 hours and the resulting products were confirmed by HPLC and LC-MS to be cycl-SNAF2-BDP or cycl-SNAF4-BDP (Figures S4). The size of the nanoaggregates was measured by DLS and TEM to be around 300 nm for both cycl-SNAF2-BDP and cycl-SNAF4-BDP (Figure 3b-e).

To evaluate if B-SNAF2/4-BDP was selectively retained in LacZ-expressing living cells, we tested them in 9L rat gliosarcoma cells transfected with LacZ (9L/LacZ). LacZ expression in 9L/LacZ cell line was confirmed by X-Gal staining, a widely used colorimetric assay to detect β -galactosidase activity (Figure S5). Both 9L/LacZ and control 9L cell lines (9L/Luc expressing luciferase) were incubated with B-SNAF2-BDP or B-SNAF4-BDP (2 μ M at 37 °C for 2 hours) followed by nuclear staining with Hoechst. When incubated with B-SNAF4-BDP, 9L/LacZ showed 2.5-fold higher retention of fluorescent signal than 9L/Luc (Figure 3f and S6). For B-SNAF2-BDP, 9L/LacZ only showed slightly higher (1.3-fold) fluorescent signal than 9L/Luc (Figure 3g and S6). In addition, the uptake of B-SNAF2-BDP in both 9L/LacZ and 9L/Luc were higher than with B-SNAF4-BDP, which may come from higher hydrophobicity of B-SNAF2-BDP and stronger non-specific retention.

Imaging of Proteases Activity in Apoptotic Cells.

We have previously applied the CHQ based scaffold to image therapy-induced tumor apoptosis.^[17] We next validated that SANT4 could be applied to image caspase-3/7 activity in human lung cancer. We synthesized the caspases-sensitive nano-aggregation fluorescent probe C-SNAF4-Cy5 with caspases-3/7 as the target. DEVD, a short peptide substrate of the endopeptidase caspase-3/7, and disulfide incorporated into the new scaffold as the masking groups (Figure 4a).^[17] After cleavage of the DEVD by caspases with the presence of glutathione in cells, intramolecular cyclization proceeds between the terminal cysteine and aromatic nitrile will produce Cy5-labeled macrocyclics to afford cycl-SNAF4-Cy5. We validated the probe mechanism *in vitro* first by incubating C-SNAF4-Cy5 with human recombinant caspase-3 in caspase buffer. The macrocyclic product from the enzymatic reactions were analyzed by HPLC (Figure S7a) and LC-MS (Figure S7b). DLS and TEM analysis of C-SNAF4-Cy5 (20 μ M) following incubation with caspase-3 in caspase buffer overnight further confirmed the in-situ formation of nanoaggregates with an average size of around 100 nm (Figures 4b-c). To induce elevated expression of caspase-3 in cells, H460 human non-small cell lung cancer cells were treated with cisplatin (10 μ M) for 24 hours following incubation with C-SNAF4-Cy5 (2 μ M) at 37 °C for 24 hours. Caspase expression in H460 cells after cisplatin treatment was confirmed by Western blot analysis showing elevated level of caspase-3 expression after cisplatin treatment (Figure S8). The retention of C-SNAF4-Cy5 in cisplatin treated H460 apoptotic cells was further imaged by fluorescence microscopy. As shown in Figures 4d, the probe accumulated extensively in cisplatin-treated apoptotic cells, while negligible fluorescence was observed in viable cells without cisplatin treatment. Fluorescence abolishment after pan-caspase inhibitor Z-VAD-fmk (50 μ M) treatment further confirmed the activation of C-SNAF4-Cy5 by effector caspases. We also tested the toxicity of C-SNAF4-Cy5 in healthy and apoptotic H460 cells using MTS assay. In healthy cells, C-SNAF4-Cy5 did not produce toxicity up to the highest concentration (10 μ M) we tested (Figure S9). In drug-treated apoptotic cells, the compound did not induce significant added toxicity (Figure S9).

Conclusion

In this work, we step-by-step interrogated the structure-activity relationship of aromatic nitriles and aminothiols and investigated the structural parameters of small molecules that are capable of undergoing target-activated macrocyclization followed by self-assembly to form nanoparticles. Additional electron delocalization provided by added aromatic ring, electron-withdrawing substituents, and heteroatoms on the aromatic rings have substantial effects on the reactivity of aromatic nitriles for their condensation with aminothiols. We have shown that the condensation between aromatic nitriles and aminothiols can proceed intramolecularly to form macrocyclics with various ring sizes. These rigid, hydrophobic macrocyclics can further self-assemble into nano-aggregates at a size of around 100 to 250 nm. This work demonstrates that besides the CBT and luciferin moiety, other similar aromatic structures can also undergo macrocyclization and nano-assembly.

To apply the SNAT scaffold to develop a cell imaging probe, both the reaction rate of the aromatic nitrile and the ability in nano-assembly of macrocyclics are important. Other

factors like the hydrophobicity also contribute to the performance, as shown by SNAT4 that displayed less non-specific uptake and better contrast than SNAT2 in imaging β -galactosidase activity in LacZ-expressed living cells. The imaging application demonstrated in this work includes fluorescence imaging of protease and galactosidase. By changing the group attached to the cysteine residue in the scaffold, similar probes may be designed to image different enzyme targets. In addition, other imaging tags besides fluorophores may be introduced to the pyrimidine-based scaffold for other imaging modalities, like photoacoustic imaging, magnetic resonance imaging and positron emission tomography imaging. These features further demonstrate the general applicability of this imaging strategy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Analysis of cyclization reaction rates and their cyclic products. (a) Analysis of cyclization reaction by modulating condensation reaction substrate pair, different linkers and different ring sizes. 1st Order rate constants for the intramolecular cyclization were measured in PBS buffer at room temperature using HPLC assay. ^aThe rate constant was reported in Reference^[7]. (b) Dynamic light scattering (DLS) analysis of the hydrodynamic radius of I to VI and nano-aggregates of cycl-I to cycl-VI.

Figure 2.

Stability of SNAT2/4 and their cyclization products in cell lysate. (a) In-gel fluorescence and Coomassie stain analysis of SNAT2 (lane 1) and SNAT4 (lane 2) after they were incubated in HeLa lysate (37 °C, 6 hours) followed by click reaction with Cy5-azide. BSA was first labeled by 6-heptynoic NHS ester before subjecting to click reaction with Cy5-azide as a positive control (lane 3). (b) HPLC analysis of HeLa lysate after incubation of disulfide caged SNAT4 in live HeLa cells (37 °C, 6 hours). HeLa lysate without (bottom) and with (top) incubation with disulfide caged SNAT4. (c) HPLC analysis of HeLa lysate after incubation of disulfide caged SNAT4 in HeLa lysate (37 °C, 6 hours). HeLa lysate without (bottom) and with (top) incubation with disulfide caged SNAT4. * Cyclized product.

Figure 3.

In vitro and *in cellulo* validations of β -galactosidase imaging probes. (a) Proposed β -galactosidase and reduction-initiated conversion of B-SNAF2/4-BDP into B-SNAF2/4-BDP-aglycone and cycl-SNAF2/4-BDP via enzyme activation and intramolecular cyclization followed by self-assembly into nanoaggregates in situ. (b-e) DLS and TEM analyses of cycl-SNAF2-BDP (b and c) or cycl-SNAF4-BDP (d and e) following β -galactosidase activation *in vitro*. (f) Live cell imaging of β -galactosidase activity in LacZ-transfected 9L cells using B-SNAF2/4-BDP (2 μ M, 2 hours). Cells were stained with nuclear dye Hoechst 33342 (blue). Scale bar: 50 μ m. (g) Flow cytometry analysis of 9L/LacZ and 9L/Luc cells stained with B-SNAF2-BDP or B-SNAF4-BDP.

Figure 4.

In vitro and *in cellulo* validations of caspases -3/7 imaging probes. (a) Proposed caspase-3 and reduction-initiated conversion of C-SNAF4-Cy5 into cycl-SNAF4-Cy5 via intramolecular cyclization, followed by self-assembly into nanoaggregates *in situ*. (b-c) DLS and TEM analyses of cycl-SNAF4-Cy5 following caspase-3 activation *in vitro*. (d) Cell imaging of caspase-3 activity in cisplatin-induced (10 μM , 24 hours) H460 apoptotic cells using C-SNAF4-Cy5 (scale bar: 20 μm). H460 cells were treated with or without cisplatin or with additional caspase inhibitor Z-VAD-fmk (50 μM). Cells were stained with nuclear dye Hoechst 33342 (blue).

Scheme 1.

Biocompatible condensation reaction between CBT and cysteine, and its application to self-assembly by intermolecular polymerization and intramolecular cyclization.

Table 1.

Condensation reaction rate constants between the aromatic nitriles and aminothiols.

PBS, pH = 7.4
TCEP, NaHCO₃
2nd order rate constant k

Entry	Aromatic nitriles	Aminothiols	2 nd order rate constant k (M ⁻¹ s ⁻¹)
1			2.9 ± 0.3 ^a
2			0.06 ± 0.02
3			No Rxn ^a
4			2.21 ± 0.04
5			1.0 ± 0.2
6			0.010 ± 0.002
7			0.029 ± 0.003
8			5.4 ± 0.8

2nd Order rate constants were measured in PBS buffer at room temperature using HPLC assay.^aThe rate constants were reported in Reference¹³.