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Note

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Thioimidate Bond Formation between Cardiac Troponin C and Nitrile-containing Compounds

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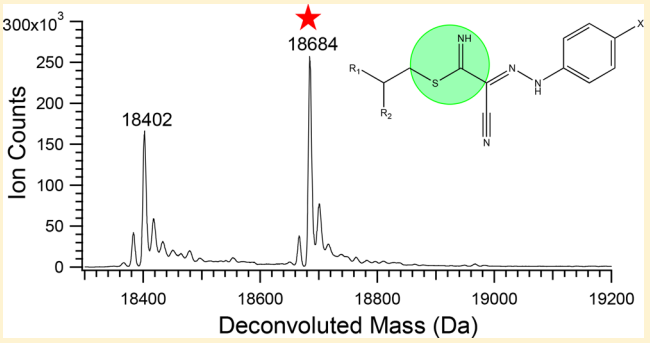
Supporting Information

ABSTRACT: We have investigated the mechanism and reactivity of covalent bond formation between cysteine-84 of the regulatory domain of cardiac troponin C and compounds containing a nitrile moiety similar to the calcium sensitizer levosimendan. The results of modifications to the levosimendan framework ranged from a large increase in covalent bond formation to complete inactivity. We present the biological activity of one of the most potent compounds. Limitations, including compound solubility and degradation at acidic pH, have prevented thorough investigation of the potential of these compounds. Our studies reveal the efficacious nature of the malononitrile moiety in targeting cNTnC and its potential in future cardiotonic drug design.



KEYWORDS: *Cardiac troponin C, reversible thioimidate bond formation, levosimendan, malononitrile*

The use of nitrile functionalities in pharmaceutical agents has become increasingly popular over the last two decades as numerous studies assessing the reactivity[1−3] and predicting biological affinity[4] of nitriles have emerged. A recent review of nitrile containing compounds that are actively being used or investigated as clinical candidates showcased the prevalence of this functionality in drug design.[5] This review included a handful of compounds that are currently utilized in the treatment of cardiovascular conditions, and of those mentioned, levosimendan is the only cardiovascular agent that is also classified as a calcium (Ca^{2+}) sensitizer. Ca^{2+} sensitizers are therapeutic agents that were developed to treat systolic heart failure. Conceptually, these compounds act directly on sarcomere proteins to modulate cardiac contraction without altering Ca^{2+} homeostasis. Since Ca^{2+} regulation is intricately coupled with other signaling pathways, the development of compounds that act purely as Ca^{2+} sensitizers is difficult. Nonetheless, development of compounds that act predominantly as Ca^{2+} sensitizers are appealing as they should reduce the adverse effects associated with prolonged use of the "calcium modulators"[6,7] that are currently administered in clinical settings.

Levosimendan is one of three Ca^{2+} sensitizers that has undergone clinical trials and remains widely used to treat patients across the world.[8] Although levosimendan does not act solely as a Ca^{2+} sensitizer, its positive inotropic effects are considered to be a result of the interactions it has with cardiac troponin (cTn).[9−12] cTn is a component of the thin-filament, which regulates cardiac contractility. cTn has three subunits (cTnC, cTnI, and cTnT), each with a different role. Of particular interest to our studies is the regulatory N-domain of cTnC (cNTnC) that binds Ca^{2+}. The binding of Ca^{2+} causes the N-domain to shift to an open state, which promotes all other conformational changes required to generate contractile force.[13,14] Understanding how levosimendan interacts with cNTnC has been of great interest for the purpose of designing more effective sensitizers that target this region.

A mechanism of action for the interaction of levosimendan with cNTnC has only recently been elucidated. This built upon existing data that indicated the integral role of cysteine-84 (C84) in levosimendan binding.[15,16] Wild-type cTnC contains two cysteines, both located in the N-domain, that are conserved across multiple species but are absent from fast skeletal isoforms of TnC. This distinction between the otherwise structurally similar Ca^{2+} binding proteins poses an intriguing avenue for tailoring compounds that react specifically with cTnC. Characterization of a levosimendan analog bound irreversibly to C84 demonstrated the advanta-

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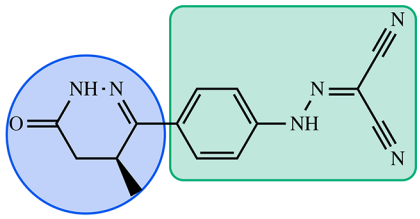
geous effects covalent modifications to cTnC had on contractile force.[17] Subsequently, the formation of a reversible covalent thioimidate bond between C84 and the malononitrile group of levosimendan was observed both *in vitro*[18,19] and *in situ*.[19]

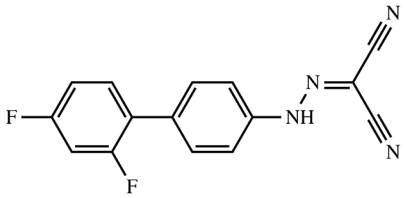
In this Note, we discuss how a selection of compounds bearing structural similarities to levosimendan (Figure 1) influenced covalent bond formation with cNTnC. We demonstrate that the covalent adducts are likely thioimidate bonds formed through a Pinner-like mechanism[2] and show that preferential binding to C84 is preserved.[18] We also present ATPase activity studies that indicate that one of the more efficacious analogs has a sensitizing effect on cardiomyofibrils. Lastly, we examine some of the obstacles encountered while using this panel of compounds.

1



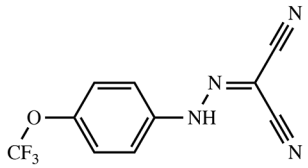
((4-(1,4,5,6-tetrahydro-4-methyl-6-oxo-3-pyridazinyl)phenyl)hydrazono)propanedinitrile

2



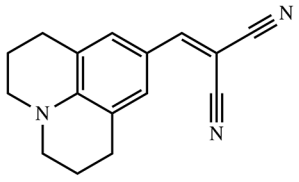
((4-(2,4-difluorophenyl)phenyl) hydrazono)propanedinitrile

3



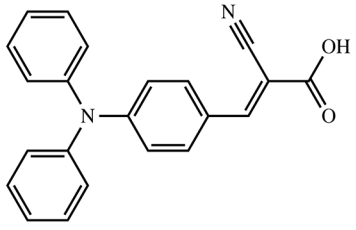
carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone

4



9-(2,2-Dicyanovinyl)julolidine

5



4-(diphenylamino)phenylcyanoacrylic acid

Figure 1. Nitrile compounds studied: levosimendan-**1**, DLEV-**2**, FCCP-**3**, DCVJ-**4**, and DNFO1-**5**.

To investigate how structural variations to the levosimendan framework influenced reactivity with cNTnC, we studied a selection of nitrile containing compounds (Figure 1). Three of these compounds had malononitrile functionalities like levosimendan (**1**), and one compound contained a cyanoacrylic acid functional group. The first compound, **2**, was synthesized in house (Scheme S1) and had an identical difluorobiphenyl backbone as two other levosimendan analogs studied.[17,20] Both of these analogs have a calcium sensitizing effect when bound to cTnC. The solution structure shows that the difluorobiphenyl backbone orients itself within the hydrophobic pocket of cNTnC,[17,20] which is thought to be important for stabilizing the open conformation of cNTnC. Another fluorine containing compound, **3**, was investigated. Compound **3** is a toxic uncoupling agent and is one of the few molecules commercially available with the same electrophilic moiety as levosimendan. Both **2** and **3** contain identical (phenylhydrazono)malononitrile functionalities to levosimendan (**1**, green rectangle).

An additional compound with a malononitrile moiety was selected for our reactivity experiments. Compound **4** differs as it contains a methylene, rather than a hydrazono, connection between the aromatic and malononitrile groups. In the past, **4** has been used to label calmodulin,[21] another EF-hand protein that binds Ca^{2+}. The final compound we explored had a phenylcyanoacrylic acid functionality connected to a diphenylamine group (**5**). High affinity binding has been reported for small diphenylamine-containing compounds to a chimeric cTnC−cTnI complex.[22] Solution structures indicate that these compounds bind deep within the hydrophobic pocket of cNTnC.[22] Compound **5** allowed us to explore if high affinity binding with cNTnC would be observed with larger diphenylamine compounds and provided us with a means to investigate if cyanoacrylic acids reacted with thiols in the same manner as compounds with the malononitrile functionality.

All of the spectroscopic experiments were conducted using recombinant human cTnC, $cTnC_{C35S}$, $cNTnC_{C35S}$, or $cNTnC_{C35S,C84S}$. These proteins were expressed and purified according to previously established protocols.[22,23] The molecular weights of these proteins are 18402, 18387, 10925, and 10910 Da, respectively, and were verified by mass spectrometry (MS). Both cNTnC constructs have an extra glycine (G90) due to the cloning process and contain a C-terminal histidine tag (His-6X) for purification purposes.

The reactivity of the compounds was evaluated in time course experiments, where the amount of covalent species formed over 5 h was monitored by reverse phase high performance liquid chromatography mass spectrometry (RP-HPLC-MS). These *in vitro* experiments were performed under the same conditions as past experiments,[18,19] where 1 mg/mL stocks of protein were prepared the day of experiments using an aqueous buffer comprising 100 mM KCl, 10 mM imidazole, and 200 μM Ca^{2+} at pH 7. Stock solutions of the compounds were prepared in reagent grade DMSO and aliquoted into each of the 50 μL samples to obtain 50 μM of the desired compound (0.5−2.5% v/v DMSO) in solution. For the time-course experiments in this Note, we evaluated covalent bond formation exclusively between $cNTnC_{C35S}$ and each compound at a single concentration. The data collected from the time-

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course experiments were used to construct plots of the fraction of thioimidate species formed over the total amount of unreacted and reacted cNTnC$_{C35S}$ (Figure 2).

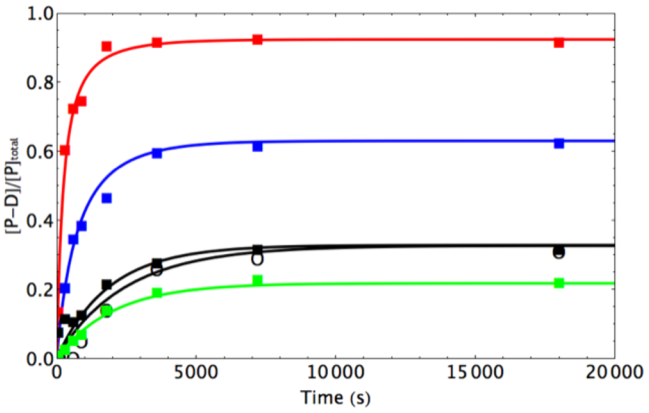

Figure 2. Formation of a covalent species with 1 mg/mL cNTnC$_{C35S}$ (squares) or cTnC$_{C35S}$ (circles) monitored by RP-HPLC-MS:[18,19] 50 μM **3** (red), **2** (blue), levosimendan (black), and **5** (green) reacted with cNTnC$_{C35S}$ over 5 h. Black line through circles corresponds to previously reported data for 50 μM levosimendan reacted with cTnC$_{C35S}$.[18,19]

The covalent species formed between cNTnC$_{C35S}$ and compounds **1**, **2**, **3**, or **5** had molecular weights of 11206, 11207, 11179, and 11266 Da, respectively. Mathematica was used to fit these points to a reversible covalent mechanism (Scheme S2) using the same process described in Klein et al.[19] Utilizing this fitting process allowed for the extraction of rate constants that provided the best fit for each of the compounds (Table 1). Compound **4** was excluded from these experiments

Table 1. Rate Constants Used To Fit cNTnC$_{C35S}$ (Squares) Curves in Figure 2

compounds	k_1 (M^{-1} s^{-1})[a]	k_{-1} (s^{-1})	k_2 (s^{-1})	k_{-2} (s^{-1})
3 (red)	1×10^7	1500	0.018	0.00001
2 (blue)	1×10^7	7500	0.016	0.00001
1 (black)	1×10^7	7500	0.002	0.0003
5 (green)	1×10^7	5	0.0002	0.0003

[a]Values are fixed.

as no discernible amount of covalent species was detected in the mass spectrum when it was reacted overnight with cTnC$_{C35S}$ (unpublished data). This result was surprising considering **4** has a malononitrile group and has been used in fluorescent studies with calmodulin. It is possible that the bulky aromatic group inhibits **4** from entering the hydrophobic cleft, preventing the proximity necessary for it to react with C84.

Our data show that the amount of covalent species detected between cNTnC$_{C35S}$ and the malononitrile containing compounds increased considerably as the size of the aromatic regions was reduced. In comparison, a decrease in the amount of covalent adduct was observed when **5** was reacted under the same conditions as the malononitrile compounds (Figure 2). This suggests that the nitrile of the cyanoacrylic acid group is less reactive or the larger ring system slows compound **5** from entering the hydrophobic pocket. The time-course data for 50 μM levosimendan reacted with cTnC$_{C35S}$ (Figure 2, black circles) was obtained from experiments conducted previously.[18] The rates used to fit 50 μM levosimendan reacted with cNTnC$_{C35S}$ (Table 1) are virtually identical to the rates reported for this reaction with cTnC$_{C35S}$.[19] This indicates that the presence of the C-domain does not alter thioimidate bond formation nor does any "off-target" binding associated with this domain.

Determination of the rate constants for these new compounds fit to a reversible covalent mechanism is underdetermined. We have therefore fixed the forward rate constant (k_1) for the formation of the Michaelis complex (P:D) to a typical diffusion-limited value. All other rates determined for compounds **2**, **3**, and **5** reacting with cNTnC$_{C35S}$ were determined from the best fit to the data (Table 1). The rate of dissociation (k_{-1}) for P:D varied between these three new compounds, with **2** having the most similar k_{-1} to levosimendan. The greatest variation in rates was seen with **5**. The changes indicates that **5** has the strongest binding affinity associated with P:D and is consistent with the reported values for other diphenylamine compounds.[22] The reversibility of the covalent species is given by the k_{-2} value and is on the order of hours for these compounds.

It is important to note that MS cannot be used alone to distinguish if the covalent species formed between these compounds and cNTnC$_{C35S}$ are thioimidate adducts. This is due to the products of Michael additions having the same overall mass change as thioimidate adducts that form via a Pinner-like mechanism. Products associated with both of these mechanisms have been observed when thiols are reacted with nitrile containing compounds. Robertson et al.[18] reported that levosimendan reacted with glutathione to form both a thioimidate and a thiazoline adduct. These adducts are the predicted products that form via a Pinner-like mechanism, where the free cysteine attacks the carbon of the nitrile.[2] Other research has reported the formation of thioether adducts when compounds with cyanoacrylate groups were reacted with a model cysteine.[24] Formation of thioethers proceeds via a Michael addition, where the model thiol reacts with the beta-carbon of the methylene group. Given the structural similarities of compounds **2** and **3** to levosimendan, coupled with the spectroscopic evidence that establishes thioimidate bond formation between C84 and levosimendan,[18] we hypothesize the covalent species formed between these two analogs and cNTnC are indeed thioimidate adducts. To investigate if compound **5** would react with a thiol to yield a thioimidate adduct, we followed the reaction of *N*-acetyl cysteine (NAC) with **5** over 24 h using 1D ^{1}H NMR spectroscopy (Figure 3).

An array of spectra was collected at 30 °C on a 600 MHz Varian spectrometer (Supplementary Methods) to monitor the reaction of **5** with NAC in deuterated DMSO (DMSO$_{d6}$). The spectra collected for this reaction show that the thiol of NAC reacts with the carbon of the nitrile on compound **5** and is supported by new resonances for all of the protons on NAC such as the NH peak arising at 10.23 ppm (Figure 3). The spectra show that the proton (H_{20}) at 8.18 ppm, assigned to the beta-carbon proton of the methylene, remains unchanged through the 24 h experiment. If the reaction between thiols and **5** proceeded via a Michael addition, we would expect the resonance position of this peak to change dramatically and a new CH peak to appear for a proton on the α carbon. The absence of these changes, along with the formation of a new NH peak, provides spectroscopic evidence that thioimidate bonds are formed between free thiols and cyanoacrylic acids,

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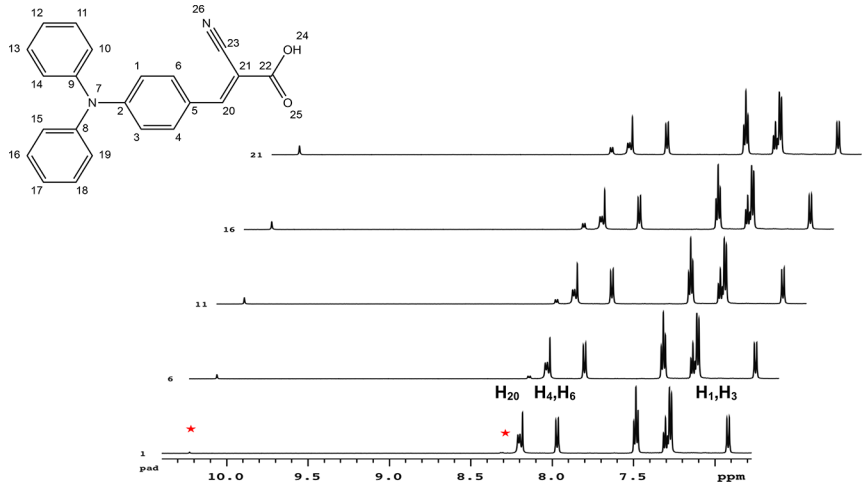

Figure 3. Reaction of 2 mM compound **5** with 4 mM *N*-acetyl-L-cysteine (NAC) in $DMSO_{d6}$ monitored by ^1H NMR at 1, 6, 11, 16, and 21 h. ^1H NMR resonances from **5** are assigned in the bottom spectrum between 6.91−8.18 ppm and remain unchanged. The unreacted amide NH peak of NAC at 8.21 ppm decreases in intensity and new NH peaks arise (indicated by stars). The NH singlet at 10.23 ppm is attributed to the formation of a thioimidate bond. The constant peak intensity of H_{20} throughout this time course experiment and lack of new CH peaks supports that a thioimidate bond formed via a Pinner reaction[2] with these compounds rather than a Michael addition.[24]

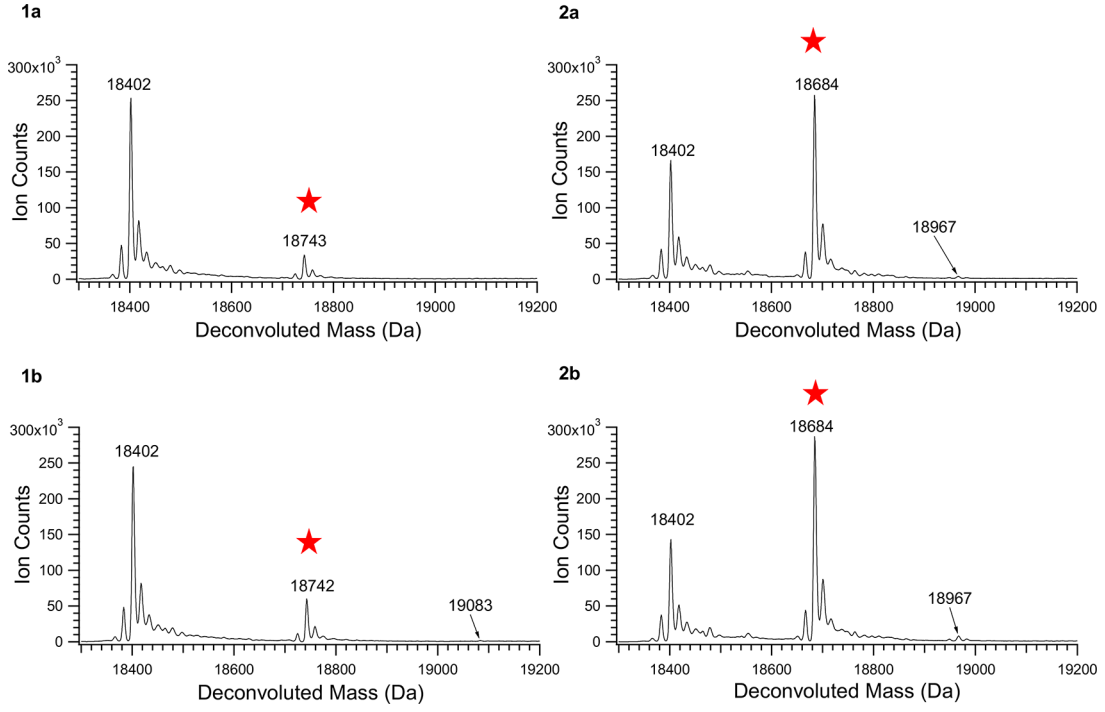

Figure 4. Deconvoluted mass spectra showing that compounds **5** (1) and **2** (2) form predominately a single thioimidate bond in the presence of wild type cTnC at 1:1 (a) and 2:1 (b) ratios. The preferential reaction of levosimendan for C84 was demonstrated in previous work.[18] Masses corresponding to the thioimidate species are 18743 Da for cTnC-**5** and 18684 Da for cTnC-**2** (red stars). Minor amounts of another thioimidate species can be observed at 19083 or 18967 Da after 24 h.

even when a methylene group is present. An analogous experiment with NAC and levosimendan was preformed,[25] and the rates are comparable to the reaction of **5** with NAC (data not shown). This supports that cyanoacrylic acids and malononitriles have similar reactivity toward free thiols.

Our spectroscopic data supports that the covalent species observed in the mass spectrometry experiments with $cNTnC_{C35S}$ are thioimidate adducts. To examine if these compounds preferentially reacted with C84 like levosimendan, we reacted **2** and **5** with wild-type (wt)-cTnC and monitored the formation of the thioimidate species using mass spectrometry. Levosimendan was shown to selectively react with C84, with no indication of a thioimidate species forming once C84 was mutated out of the cTnC construct.[18] The two cysteines present in wt-cTnC are both located in the N-domain. C35 composes part of the first beta-strand, and C84 is positioned at the end of helix D, near the opening of the hydrophobic pocket. Compounds **2** and **5** were both mixed with wt-cTnC overnight to obtain a 1:1 and 2:1 ratio of the molecules to cTnC (Supplementary Methods).

At both concentrations, compounds **2** and **5** predominantly formed a single thioimidate species with cTnC (Figure 4).

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Small amounts of a secondary covalent species were observed and have a corresponding change in mass consistent with a second thioimidate species being formed with C35. This amount of secondary thioimidate species is negligible provided that formation occurs after 18+ hours of incubation. These nitrile containing compounds preserve the preferential reaction with C84 and suggest that these nitrile functionalities are advantageous and should be explored further in the design of new cardiotonic drugs.

To assess whether the more active analogs were calcium sensitizers, actomyosin ATPase activity measurements were conducted with bovine cardiomyofibrils (CMFs) for compound **2**. Our studies were performed at a physiological Ca^{2+} concentration of 1 μM, an appropriate concentration to mimic systole with compound **2** (Figure S3). Additions of **2** to the CMFs caused an increase in ATPase activity. This result indicates that **2** has a calcium sensitizing effect consistent with the other difluorobiphenyl analogs studied.[17,20] These experiments substantiate the effective role of covalent modification to C84 by nitriles.

Further insights into the interactions these compounds have with cTnC have been hindered by compound solubility. We've attempted to determine the binding affinities of these compounds using 1D ^{1}H and 2D ^{15}N,^{1}H HSQC NMR experiments (unpublished results). Optimized conditions for these 2D NMR experiments require a relatively high protein concentration and slightly acidic pH. This has limited our investigation into the binding of compounds **2** and **5** with cTnC, as both have low solubility in aqueous solution at physiological pH (<500 μM). We also observed **2** was acid labile (Figure S5), making it difficult to maintain pH during titrations.

In this Note, we have demonstrated that alterations in the levosimendan framework can tune the reactivity of nitrile compounds for cNTnC. We have shown that these molecules target cNTnC directly through thioimidate bond formation with C84. We suggest that the development of a wider selection of compounds that contain the malononitrile or cyanoacrylic acid functionality appears to be a promising avenue for cardiotonic drug design. The design of new compounds with these functionalities should consider the addition of substituents that aid in the solubility of these compounds at physiological pH.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmedchemlett.9b00168.

Protocol for the synthesis of compound **2**, protocols for NMR, RP-HPLC-MS, and ATPase activity experiments, and reaction scheme for formation of a reversible covalent bond (PDF)

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS

cTn, cardiac troponin; cTnI, cardiac troponin I; cTnT, cardiac troponin T; cNTnC, N-domain of cardiac troponin C; HSQC, heteronuclear single quantum correlation

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