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Computational studies on the Carboni-Lindsey reaction

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Running Title: *DFT studies on the Carboni-Lindsey reaction*

Abstract

The Carboni-Lindsey reaction between tetrazines and strained unsaturated hydrocarbons has in recent years become a useful tool in bioorthogonal chemistry for labelling biomolecules *in vitro* and *in vivo*. Here, we report computational studies on the mechanism of the *Diels-Alder/Retro-Diels-Alder* reaction between a commercially available tetrazine and norbornene. Isomerism of the reaction with a norbornene-amide relevant for applications in bioconjugation chemistry, as well as the role of protonation in the mechanism, is assessed.

Key words: Carboni-Lindsey reaction, density functional theory, reaction mechanisms, copper-free “click” chemistry, tetrazine.

1. Introduction

In the 1950s, Carboni and Lindsey reported experimental studies on the synthesis[1] and reactivity of 3,6-disubstituted symmetric tetrazines with a series of alkenes (Scheme 1).[2] These reactions are spontaneous and proceed *via* an exothermic 1,4-cycloaddition at room temperature leading to the release of nitrogen gas and the formation of either dihydropyridazines or the oxidised 3,4,5,6-tetrasubstituted pyridazine compounds. Since these early reports, there has been a sustained interest in adapting this reaction for use in synthesis,[3–7] and more recently in the bioorthogonal labelling of proteins *in vitro*[8–13] and *in vivo*. [14–25] In addition, Schirmmayer and co-workers have adapted the tetrazine-labelling technology as a facile route for covalent modification of highly-ordered graphitic surfaces.[26] The topic of bioorthogonal ligation using tetrazine-based reagents (amongst others) has featured in a number of excellent reviews.[27–38]

Experimental properties of tetrazine reactions with different dienophiles have been studied in detail whereby reaction rates are found to vary with the electronic nature of the substituents on both the 3,6-disubstituted tetrazine and the dienophile. In most cases, a frontier molecular orbital (FMO) description of the rate-determining [4+2]-cycloaddition step is sufficient to explain the experimentally observed reactivity. Electron donating groups (EDGs) in the 3- and 6-positions of the tetrazine combined with electron withdrawing groups (EWGs) on the dienophile favour a ‘normal’ Diels-Alder description involving interaction of the tetrazine highest occupied molecular orbital (HOMO) with the low-lying lowest unoccupied molecular orbital (LUMO) of the dienophile.[35] Switching the polarity of the substituents by inserting EWGs on the tetrazine, or destabilising the HOMO of the dienophile with EDGs or the use of highly strained ring systems leads to a facile reaction through an inverse electron-demand Diels-Alder (IEDDA) pathway. Where dienophile reagents with strained rings are employed, the alternative name of

strain-promoted inverse electron-demand Diels-Alder (SPIEDDA) has been used to describe the chemistry.

From a computational perspective, many groups have investigated the molecular features and electronic structure properties of the tetrazine-dienophile reaction.[39–47] The majority of studies focused on the role of different substituents on accelerating the reaction rates through control of the relative FMO energies. Houk and co-workers used an energy decomposition distortion/interaction model (DIM)[46] to investigate the effects of EWGs on the tetrazine, concluding that experimental reactivity correlates with the electron-withdrawing character of the substituents. In 2009, Domingo *et al.* investigated the ‘unexpected’ regioselectivity of this hetero-Diels-Alder with tetrazines and electron rich alkenes using density functional theory (DFT; B3LYP/6-31G(d)).[42] Regioselectivity was explained in terms of a polar cycloaddition model.[47] Expanding on this approach, Domingo *et al.* recently applied the Molecular Electron Density Theory (MEDT)[48] approach to study correlations in the global electron density transfer (GEDT) in the transition state structures and activation thermodynamics for the reactivity of 3,6-disubstituted tetrazines with tetramethylethene and tetracyanoethene.[45]

In each of these computational reports, the theoretical reaction coordinates focused exclusively on the direct reactivity of the tetrazine with the dienophile. In this work, we investigated the regioselectivity and energetics for reactions between a monofunctionalised 6*H*-tetrazine with the aryl substituent $-\text{C}_6\text{H}_4\text{CH}_2\text{NH}_2$ in the 3-position with norbornene derivatives. The choice of reagents reflects one of the bioorthogonal pairs that have shown promise for labelling biomolecules *in vitro* and *in vivo*. [9,14]

2. Computational Details

All calculations were conducted by using density functional theory (DFT) as implemented in the Gaussian16 Revision A.03 suite of *ab initio* quantum chemistry programs.[49] Normal self-consistent field (SCF) and geometry convergence criteria were employed throughout. Structures were optimised *in vacuo* without the use of symmetry constraints (C_1 -symmetry). Harmonic frequency analysis based on analytical second derivative was used to characterise optimised gas phase structures as local minima on the potential energy surface. Structures were optimised using the first-generation hybrid exchange-correlation (XC) functionals B3LYP combined with the all-electron triple- ζ Pople basis set and 6-311++G(d,p).[50] Solvation effects were incorporated iteratively by performing self-consistent reaction field (SCRF) calculations using the integral equation formalism polarisable continuum model (IEFPCM) initially developed by Tomasi and co-workers.[51] The solute-solvent boundary was defined by using a solvent excluding surface (SES). The molecular solute surface was defined by using the United Atom Topological model (UAHF) for the radii of the solute atoms. Default settings were employed in the calculation of solvation free energies, $\Delta G_{\text{sol}} / \text{kJ mol}^{-1}$. A water solvent continuum model with dielectric constant, $\epsilon = 78.3553$, and solvent sphere radius, $R_{\text{solv}} = 1.385 \text{ \AA}$. The choice of solvation model reflects the situation encountered when performing pre-targeting *via* ‘click’ labelling methods *in vivo*. Optimised structures were analysed by using Chemcraft (version 1.8, build 536b).[52]

3. Results and Discussion

3.1 Reaction coordinates for *exo*- and *endo*-attack

In 2008, Devaraj *et al.* used a diastereomerically pure norbornene derivative, (1*S*,2*S*,4*S*)-bicyclo[2.2.1]hept-5-ene-2-yl acetic acid, as their strained dienophile in a reaction with a tetrazine (Scheme 2). Four distinct products with different yields and distinct retention times

were identified by liquid chromatography-mass spectrometry (LC/MS).[9] To investigate the regioselectivity with respect to the *exo*- or *endo*- attack of the bicyclic arene at the tetrazine, we calculated the energetics for the sequential hetero-Diels-Alder, retro-Diels-Alder reaction between the aryl-substituted tetrazine and non-functionalised norbornene (Scheme 3). Reaction coordinates for the *exo*- and *endo*-pathways are presented in Figure 1 (further details are given in the supporting information Table S1). For both pathways, the rate determining step was correctly identified as the [4+2]-cycloaddition whereby the first transition state on the *exo*-pathway (*exo*-TS1) was found to be 39 kJ mol⁻¹ lower in the calculated free energy than the corresponding *endo*-attack. In both pathways, a bicyclic intermediate was identified for which the *exo*-adduct was calculated to be slightly lower in free energy than the *endo*-adduct by 13 kJ mol⁻¹. For each of these two intermediates, the retro-Diels-Alder step leading to the release of dinitrogen can occur *via* two distinct transition states. Dinitrogen can be released from either the nitrogen pair located in an ‘axial’ (ax; parallel to the norbornene) or an ‘equatorial’ (eq; distal to the norbornene) position. For both the *exo*-adduct and the *endo*-adduct, release of dinitrogen from the ‘equatorial’ position was calculated to be thermodynamically favoured over the axial N₂ release by between 30 and 38 kJ mol⁻¹. The lowest free energy barriers to form the ‘equatorial’ transition state 2 for dinitrogen release were only +13 kJ mol⁻¹ and +8 kJ mol⁻¹ higher than the adducts for the *exo*- and the *endo*-pathways, respectively. In both cases, spontaneous release of dinitrogen is predicted to occur at ambient temperature with the reaction driven by both enthalpic and entropic factors (Table S1). Following the loss of N₂, another intermediate species is formed whereby the *exo*-int and *endo*-int compounds are of equivalent energy. Rapid isomerisation yields the experimentally observed 1,4-dihydropyridazine products (*exo*-product and *endo*-product) which are also energetically equivalent. The conclusion from these calculations is that

while both the *exo*- and *endo*-pathways are thermodynamically feasible under ambient conditions, the *exo*-products are likely to predominate in these [4+2]-cycloaddition reactions.

3.2 Isomers of the [4+2]-cycloaddition products

To address the role of isomerism resulting from the use of a substituted norbornene starting material, we calculated the relative energies of the rate determining [4+2]-cycloaddition transition states (TS1) and the free energy of the products (Figure 2). Note that here, we used both the (1S,2S,4S)-bicyclo[2.2.1]hept-5-ene-2-yl methylamide and the (1S,2R,4S)-bicyclo[2.2.1]hept-5-ene-2-yl methylamide species ($R = -CH_2C(O)NHCH_3$, where the model amide reflects the chemistry used to derivatise a biomolecule with a norbornene) to generate a total of eight possible geometric isomers. The two lowest lying transition states were identified as the *exo-endo*-b and the *exo-exo*-b species.

Six of the eight possible transition states were calculated to lie in the relative energy range of 180 to 208 kJ mol⁻¹. The remaining two transition states, corresponding to an initial *exo*-attack of the norbornene with respect to the orientation of the bicycle giving species *exo-endo*-b-TS and *exo-exo*-b-TS were lower in energy at 141 kJ mol⁻¹ and 144 kJ mol⁻¹, respectively. As with the calculations on the reaction coordinates with the non-substituted norbornene, an *exo*-orientation of the bicycle with respect to the tetrazine is energetically more accessible. This is likely due to reduce strain from decreased steric crowding in the TS1 structures when the methylene bridge lies in closer proximity to the tetrazine than for the opposite (*endo*-attack) where the larger ethylene bridge is situated closer to the tetrazine. In three of the four lowest-energy transition states, the norbornene substituent is located in ‘position b’ on the opposite side of the molecule from the Ar substituent on the tetrazine. This is also likely the result of decreased steric crowding

in the transition state and the calculations indicate that both the (1S,2S,4S)- and (1S,2R,4S)- isomers (*exo-endo*-b and *exo-exo*-b, respectively) are energetically equivalent. For the products, six of the compounds are essentially isoenergetic with calculated overall reaction free energies between -221 to -231 kJ mol⁻¹. Two products, the *endo-endo*-a and *endo-endo*-b isomers, lie higher in energy. Given that the rate determining step is assigned to the [4+2]-cycloaddition, and the reaction mechanism involves a fast, irreversible loss of dinitrogen, it is likely that all eight possible geometric isomers would be observed in reactions that utilise a mixture of norbornene diastereomers as the starting material. These computational results are consistent with the experimental observation of four different products reported by Devaraj *et al.* when starting with a diastereomerically pure norbornene reagent (Scheme 2).

3.3 [4+2]-cycloaddition transition state geometry

Inspection of the geometry of the lowest energy transition state (*exo-endo*-b-TS) revealed that the [4+2]-cycloaddition is a concerted but asynchronous process (Figure 3). The first C-C bond is formed between the unsubstituted C6 atom of the tetrazine and the norbornene. In the optimised transition state of *exo-endo*-b-TS, the $r(\text{C6-C}_{\text{norbornene}})$ interaction has a calculated distance of 2.052 Å. In contrast, the Ar-substituted C3 atom of the tetrazine forms a considerably longer interaction with the norbornene at 2.350 Å.

3.4 Tetrazine protonation: an alternative reaction pathway?

To the best of our knowledge, almost all prior theoretical studies on the reaction between tetrazines and unsaturated systems focused exclusively on the neutral reaction pathways.[39–45] Lewis acid catalysts can also be employed to accelerate the reaction rates.[53] Given the sensitivity

of the reaction kinetics toward the electronic properties of substituents on both the tetrazine and alkene reaction partners, we hypothesised that protonation of the tetrazine might also play a role in accelerating the reaction by altering the relative energy of [4+2]-cycloaddition step (TS1). A schematic representation of the different protonation sites on the tetrazine nitrogen atoms (labelled 1 to 4) is presented in Figure 4. Optimised structures for each of the four possible transitions states for the *exo*- and *endo*-pathways, the relative change in free energy with respect to the formation of the transition state from the reactants (ΔG_{TS1} / kJ mol⁻¹), and pertinent distances between the C3 and C6 atoms of the tetrazine and the norbornene are presented in Figure 5. As can be inferred from the relative energies of the different transition states, protonation of the tetrazine on the less sterically hindered positions (corresponding to N2 and N4, located adjacent to the unsubstituted C6 atom) has little to no influence on the stability of the [4+2]-cycloaddition step. For example, for the *exo*-pathway, the non-protonated route has a calculated free energy barrier of +140 kJ mol⁻¹ (Figure 1) whereas for the two most stable protonated species (*exo*-TS-H2⁺ and *exo*-TS-H4⁺), the calculated ΔG_{TS1} was +137 and +139 kJ mol⁻¹, respectively. The same trend is observed for the *endo*-pathway with protonation at the N2 and N4 positions leading to the most stable structures but the relative difference in energy of the *endo*-TS-H2⁺ and *endo*-TS-H4⁺ species, when compared with the non-protonated *endo*-TS was <7 kJ mol⁻¹, which lies close to the error-limits of this DFT approach for determining chemical accuracy. Interestingly, protonation at the more sterically constrained positions (corresponding to N1 and N3) destabilised the transition states by 17 to 18 kJ mol⁻¹ for *exo*-TS-H1⁺ and *exo*-TS-H3⁺ and by 20 to 24 kJ mol⁻¹ for *endo*-TS-H1⁺ and *endo*-TS-H3⁺. GEDT increases with protonation from values of 0.26 and 0.25 for the *endo* and *exo* transition states to between 0.40 and 0.45 for the *endo*-pathway and between 0.37 and 0.39 for the *exo*-pathway (Table S2). This

indicates that, as expected for a cationic species, the reaction goes through a more polarised pathway when protonated. When comparing the geometries of these eight different protonated transition states, an interesting trend emerges – the most stable structures display the largest difference between the $r(\text{C3-C}_{\text{norbornene}})$ and $r(\text{C6-C}_{\text{norbornene}})$ interaction distances. The energetically preferred reaction pathway involves the highest degree of asynchronicity in the two new C-C bonds formed. Protonation, and the *endo*- versus *exo*-attack orientation has almost no effect on the initial C6-C_{norbornene} bond formation with distances in the range 2.003 to 2.076 Å. In contrast, the C3-C_{norbornene} distance varies from 2.350 Å in the non-protonated *exo*-pathway to between 2.481 to 2.824 Å for the four protonated *exo*-TS-H[1-4]⁺ species. The conclusion from the calculation of these protonated species is that while the non-protonated reaction pathway remains the most likely mechanism for reactions performed under physiological conditions (pH ~7), the potential protonation of the tetrazine under slightly acidic conditions provides an alternative reaction route but is unlikely to influence the reaction rates.

As an extension, we also investigated the influence of protonation on the second transition state corresponding to the retro-Diels-Alder step and the elimination of N₂ from the *exo*- or *endo*-adducts (Figure 6). Elimination of N₂ is both enthalpically and entropically driven, leading to highly stable products. For the non-protonated reaction pathways, the calculated transition state barrier, ΔG_{TS2} , of between 8 to 38 kJ mol⁻¹ was identified (Figure 1). In contrast, protonation at the N1 or N2 position for either the *exo*-adduct or *endo*-adduct, and also the N4 position for the *exo*-adduct induced the immediate, barrierless release of dinitrogen with no stable intermediates found on the potential energy surface. Protonation at the N3 and N4 position for the *endo*-adduct and the N3 position for the *exo*-adduct do not influence the original barrier to dinitrogen loss with the same calculated barrier to axial N2 elimination in the range of 30 to 42 kJ mol⁻¹.

4. Conclusion

The mechanism of the [4+2]-cycloaddition (Carbonyl-Lindsey) reaction between a biochemically relevant tetrazine species and a strained alkene in the form of a substituted and unsubstituted norbornene was investigated by DFT. Particular emphasis was placed on the relative stability of different potential isomers formed during the reaction mechanism and in the products. The calculations support the observation of four distinct reaction products when starting from an diastereomerically pure derivative of (1S,2S,4S)-bicyclo[2.2.1]hept-5-ene-2-yl acetic acid as reported by Devaraj *et al.*[9] This indicates that the product mixture is most likely composed of the thermodynamically favoured *exo*-species whereby steric interactions between the tetrazine and the norbornene are minimised during the rate determining [4+2]-cycloaddition transition state. In addition, an investigation of the potential reaction pathways using protonated tetrazine species was performed. The calculations indicate that protonation has no effect on the rate determining step and under acidic conditions, the reaction is anticipated to proceed with similar reaction kinetics. This prediction supports the experimental observation that the bioorthogonal ‘click’ reaction proceeds rapidly *in vivo* in tumour models that potentially exhibit acidosis with reduced pH in the extracellular matrix.[14–25]

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Graphical Abstract

Scheme 1. General scheme showing the Carboni-Lindsey reaction of tetrazine with an unsaturated alkene to yield several isomeric forms of the dihydropyridazine or the oxidised 3,4,5,6-tetrasubstituted pyridazines.[2]

Scheme 2. The [4+2]-cycloaddition reaction and 4 geometric isomers as reported by Devaraj et al.[9]

Scheme 3. Reaction scheme showing the conversion of tetrazine and non-substituted norbornene into the *exo*- and *endo*-products.

Nomenclature note: *endo/exo* refer to the geometric orientation of the norbornene with respect to the tetrazine. The terms ax = axial, and eq = equatorial; where these designations refer to the position of the norbornene moiety with respect to the dinitrogen with respect that is eliminated from transition state 2 (highlighted in blue).

Relative calculated change in free energy, $\Delta G / \text{kJ mol}^{-1}$

0

Norbornene Tetrazine

Ar = $\text{C}_6\text{H}_4\text{-CH}_2\text{NH}_2$

179

exo-TS1

140

endo-TS1

endo-adduct

51

38

endo-TS2-N₂-ax

89

exo-TS2-N₂-ax

59

endo-TS2-N₂-eq

51

exo-TS2-N₂-eq

endo-int

exo-int

-217

-222

-224

-229

endo-product

exo-product

B3LYP/6-311++G(d,p)/PCM calculated reaction coordinate

Figure 2. DFT calculated free energies for the 8 possible isomeric products (black lines) and associated rate determining transition states (red lines) for the [4+2]-cycloaddition reaction between tetrazine and a derivatised norbornene bearing an amide group ($R = -CH_2C(O)NHCH_3$). Nomenclature note: the designatory letters ‘a’ and ‘b’ indicate that the Ar- and R- groups reside on the same face or opposite face of the molecule, respectively. The first *endo/exo* assignment refers to the orientation of the norbornene bicycle with respect to the tetrazine, and the second *endo/exo* assignment corresponds to the configuration of the R-group facing either the 6-membered or the 5-membered ring, respectively. Equivalent data where $R = -CH_2CO_2^-$ are presented in Supporting Information Table S1.

Figure 3. DFT optimised geometry of the *exo-endo-b-TS* species ($R = -CH_2C(O)NHMe$) showing the difference in length for the two new C-C bonds formed between the tetrazine and the norbornene units. The [4+2]-cycloaddition reaction occurs *via* a concerted, asynchronous pathway in which the C3 atom of the diene forms an initial bond with the dienophile.

Figure 4. Schematic representation of the transition state structures of the [4+2]-cycloaddition of norbornene on a protonated, 3-substituted tetrazine. Positions 1 to 4 refer to the nitrogen atoms in the tetrazine ring where ‘H1’ indicates protonation at the N1 position.

Figure 5. Optimised structures of the four possible [4+2]-cycloaddition transition states for (A) *exo*-attack, and (B) *endo*-attack of norbornene on a protonated tetrazine. Positions 1 to 4 refer to the nitrogen atoms in the tetrazine ring where ‘H1’ indicates protonation at the N1 position.

Figure 6. The influence of protonation on the second transition state (TS2) involving retro-Diels-Alder elimination of N₂ from the *exo*-adduct or *endo*-adduct formed after the [4+2]-cycloaddition step between norbornene and the 3-substituted tetrazine.