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PAPER

Concerted, highly asynchronous, enzyme-catalyzed [4 + 2] cycloaddition in the biosynthesis of spinosyn A; computational evidence†

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A theoretical study has been carried out on model systems to study a recently reported, (*Nature*, 2011, **473**, 109) biosynthetic, [4 + 2] cycloaddition catalyzed by a stand-alone enzyme (the cyclase SpnF). It was suggested in this paper that SpnF is the first known example of a Diels–Alderase (DA). In the present study, for a model system of the substrate a transition structure was found with density functional calculations (DFT). In addition, the intrinsic reaction coordinate calculations indicated that the transition structure is that of a concerted, but highly asynchronous, DA reaction. Based on the DFT and Møller–Plesset second order calculations the activation energy was estimated to be about 15 kcal mol^{-1}. The results of a natural population analysis indicated that there is significant charge transfer in the transition state, and it is proposed that possibly the enzyme plays a dual role of not only folding the substrate into the proper conformation for the DA reaction to occur, but also lowering its activation energy by stabilization of the highly polarized transition structure.

Introduction

Many reactions used in synthetic organic chemistry have been shown to also exist in the realm of enzymatically controlled biological transformations.[1] Interestingly, only recently one of the most useful synthetic reactions, the Diels–Alder (DA) [4 + 2] cycloaddition, for the first time has been suggested by Kim *et al.*[2] to occur in an *in vitro* process catalyzed by a "stand-alone" enzyme. However there have been a number of cases reported in which a DA reaction has been proposed to be catalyzed enzymatically.[3–9] In the biosynthesis of spinosyn A it was shown unambiguously that the enzyme SpnF, a cyclase found in the insecticide *Saccharopolyspora spinosa*,[10,11] catalyzes what formally may be considered a [4 + 2] cycloaddition. Kim also states[2] that their results are "…consistent with a DA reaction; however, confirmation of this hypothesis will require demonstrating that the reaction progresses through a single pericyclic transition state…". Kelly[12] has suggested that a detailed mechanistic study of the SpnF-catalyzed reaction is needed to determine whether the reaction proceeds through intermediates. If not, then SpnF would be the first example of a 'Diels–Alderase'.

It is well established that much can be learned about reactions that are catalyzed enzymatically *in vitro* by using the tools of modern quantum mechanics.[13] This is in part because of growing evidence that for many such reactions a major role of the enzyme is to encapsulate the substrate and force it into a particular conformation that allows not only for a reaction to readily occur, but to also lead to a particular stereoisomer. For example we have previously carried out DFT on the mechanism of sesquiterpenes produced by Tobacco 5-epiaristolochene synthase (TEAS).[14] The results demonstrated that the least favorable, from an energetic point of view, product of three possible sesquiterpenes is formed to the greatest extent.[15] The best example of such an enzymatically-assisted reaction is the conversion of squalene, or its oxide, to lanosterol and the hopenes. We were able to establish theoretically that the conformer of azasqualene, which is encapsulated in hopene cyclase as reported by Schulz *et al.*,[16] is very similar in structure to a conformer of squalene itself. In addition this conformer of squalene, as based on DFT calculations, after protonation undergoes a concerted cascade of cyclizations that forms the ABC rings on the way to hopene.[17]

For the biosynthesis of spinosyn A an intermediate was identified that contains both the diene and dienophile as parts of the macrocyclic lactone **2** (see Fig. 1).[2] This intermediate is formed by the dehydration of the tetraene **1**, and is proposed to then undergo a [4 + 2] cycloaddition to produce tricyclic **3**; the latter contains the newly formed cyclohexene ring. In the present study the goal was to determine whether a concerted DA reaction occurs in this conversion of **2** to **3**.

Results and discussion

To determine whether the conversion of **2** to **3** is indeed a concerted pericyclic reaction or, in contrast a stepwise, zwitterionic

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†Electronic supplementary information (ESI) available: Reaction pathway for transition structure (**9**), tables of energies and key computational output for all systems studied. See DOI: 10.1039/c2ob25827g

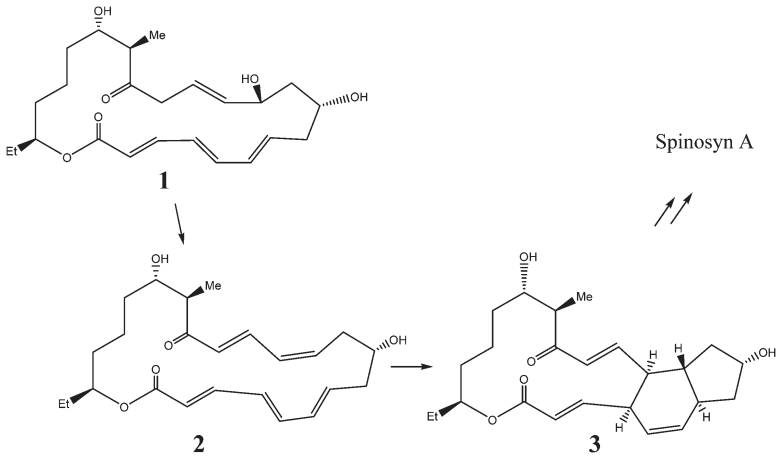

Fig. 1 Spinosyn intermediates proposed in the biosynthetic pathway.[2] An enzymatically-catalyzed dehydration of **1** yields **2**, the intermediate that undergoes the [2 + 4] cycloaddition, and yields **3**.

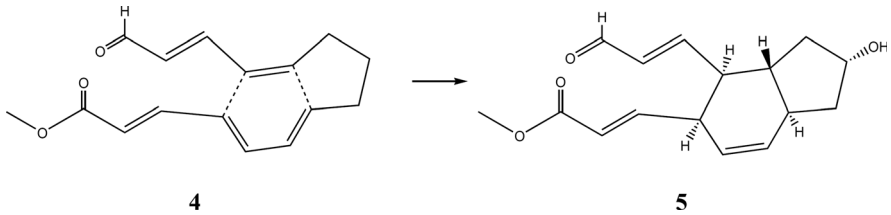

Fig. 2 Model system used to study the proposed [2 + 4] cycloaddition.

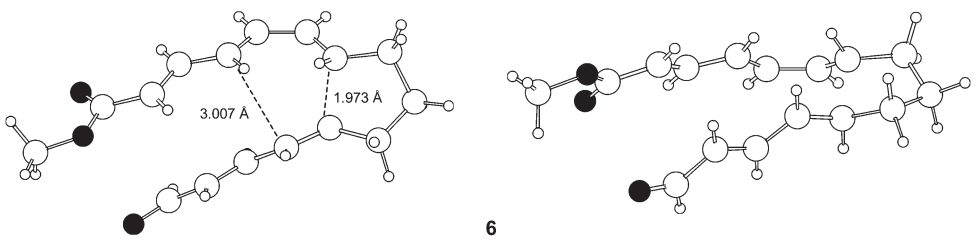

Fig. 3 Transition structure for the [2 + 4] cycloaddition. Two Chem3D views with the oxygens in black.

reaction, a model compound (**4**) was employed (see Fig. 2), which on cyclization would yield **5**. In the original system **2** five carbons of the lactone ring that contain the hydroxyl and ethyl groups, as well as the other hydroxyl group, were deleted, since it is assumed that they should not directly affect the cycloaddition reaction. In fact, the validity of this assumption is verified in the present study.

Location of the transition structure **6** in Fig. 3 was rather straightforward and was accomplished by starting with a simple DA transition structure and adding to it the appropriate, additional atoms. It is possible to deduce that this transition structure is indeed that of a DA reaction, but for a concerted, highly asynchronous process, as defined by Dewar and Pierini,[18] since in this transition structure one of the nascent sigma bonds is formed to a much higher extent than the other one. On the other hand it might also be a transition structure for a stepwise addition that would link the reactant with a zwitterionic intermediate. Dewar was the first to report experimental evidence for an asynchronous transition structure for a DA reaction for which the transition structure was unsymmetrical. Houk provided a theoretical confirmation of this in his DFT study of the reaction of isoprene with maleic anhydride.[19] In addition, theoretical studies by Sustmann and Sicking showed that DA reactions can range from synchronous to highly asynchronous concerted reactions to those that occur stepwise while passing through a zwitterionic intermediate.[20] It has also been determined that polar DA reactions do not proceed through radicaloid pathways, but rather ionic ones.[21] Hence these were not considered in the present work.

Even with the results of these previous studies it is not possible however to *a priori* decide whether **6** is the transition structure of a concerted DA reaction or that of a stepwise, zwitterionic cycloaddition leading to, or away from a zwitterionic intermediate. To distinguish between these two possibilities, an

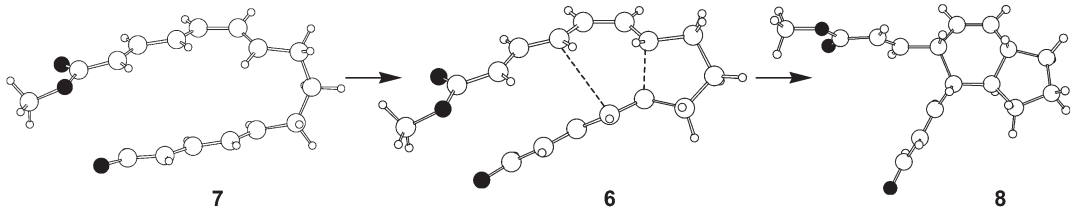

Fig. 4 Results of the IRC calculation on transition structure **6**, which was shown to link reactant **7** with product **6**. The oxygens are black.

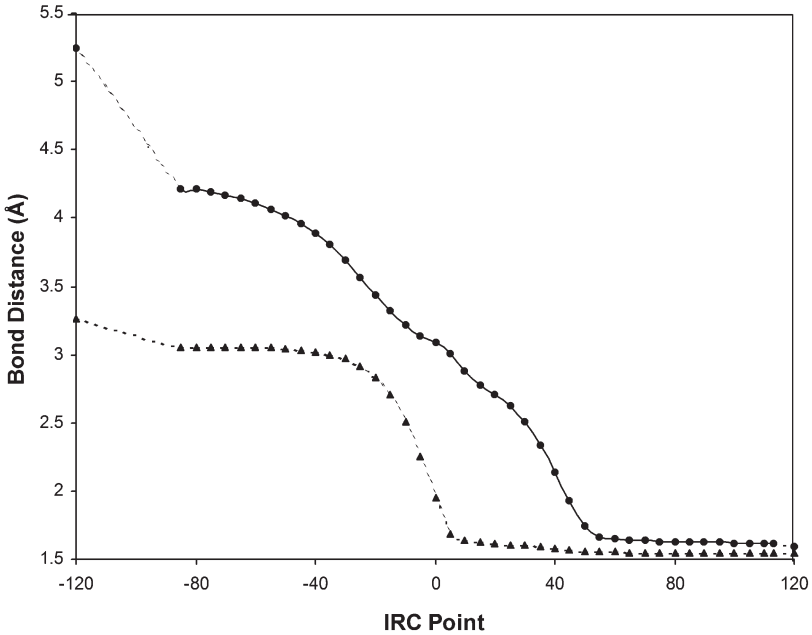

Fig. 5 A plot of the bond distances of the two forming sigma bonds *vs.* the IRC point. The points at −120 and +120 represent reactant and product respectively. Dashed lines connect the reactant and products with the point at which the IRC failed due to a very flat potential energy surface. The initially formed C–C bond is shown by ▲, and the second C–C bond by ●.

intrinsic reaction coordinate (IRC) calculation was carried out on **6**.[22,23] The results of these calculations showed that **6** indeed represents the transition structure for a concerted, asynchronous DA reaction. Indeed, it was found that **6** links directly the acyclic system **7** with the bicyclic product **8** (see Fig. 4).

In Fig. 5 the bond distances of the two forming sigma bonds are plotted against the IRC points. It is seen that the reaction is highly asynchronous, since during its course the first bond is formed much earlier than the second one. This supports the expected nature of an asynchronous DA reaction.

In Fig. 6 the energies of the structures for the corresponding points from Fig. 5 are plotted, and it is seen that there is no energy minimum along the reaction pathway, confirming that this is truly a concerted reaction.

In 1942 a seminal paper on the mechanism of the DA cycloaddition of acrolein and 1,3-butadiene Woodward alluded to the role of what is known today as a charge transfer (CT) process in the reaction.[24] More recent studies, both theoretical and experimental, have confirmed highly polarized transition structures in the case of the asynchronous DA reactions and a correlation between the degree of computed charge transfer and activation energies.[21,25] In Fig. 7 the calculated charge transfer map is given for transition structure **6**. The charge distribution evaluated with B3LYP and mPW1PW91 (in italics in the figure) indicates that there is significant CT in this transition structure. The dienophile part of **6** (top left box in Fig. 7) is calculated to have a partial negative charge, while the diene portion and the 3-carbon "tether" bear a partial positive charge, creating the polarized structure. The B3LYP CT value of 0.10 is in line with the value of 0.15 calculated for the DA reaction of acrylonitrile and cyclopentadiene.[21] Also the computed activation energy of 21 kcal mol^{-1} with B3LYP is similar to the activation energy computed for the reaction of cyclopentadiene and acrylonitrile (18 kcal mol^{-1}).[21] Given this result, we suggest that the enzyme stabilizes the charge separation created during the course of the reaction. This could significantly lower the activation energy in relation to that calculated for the gas phase (for further discussion see below). F. E. Michael as reported in Chemical & Engineering News in May of 2011 raised the interesting question of how nature might accelerate a DA cyclization.[26] Further experimental studies are required, *e.g.*, the X-ray structure of SpnF, before our results answer this question for sure.

Of course there are potentially numerous conformers of **6**. However, for two reasons the number of potential conformers of **6** that need to be considered is significantly less. Firstly, presumably the 1,3-diene and 1,3,5-triene systems in the lactone **2** have

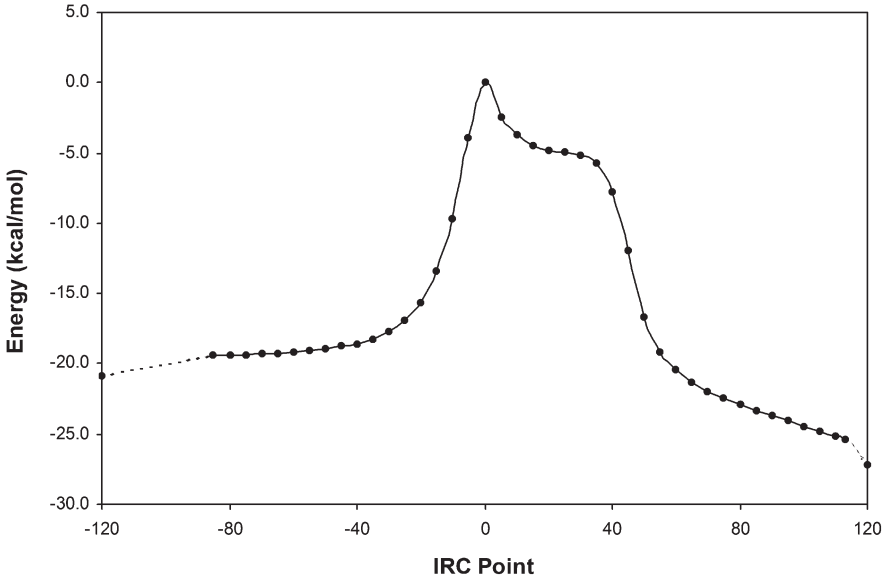

Fig. 6 A plot of the energies of the IRC points. The points at −120 and +120 represent reactant and product.

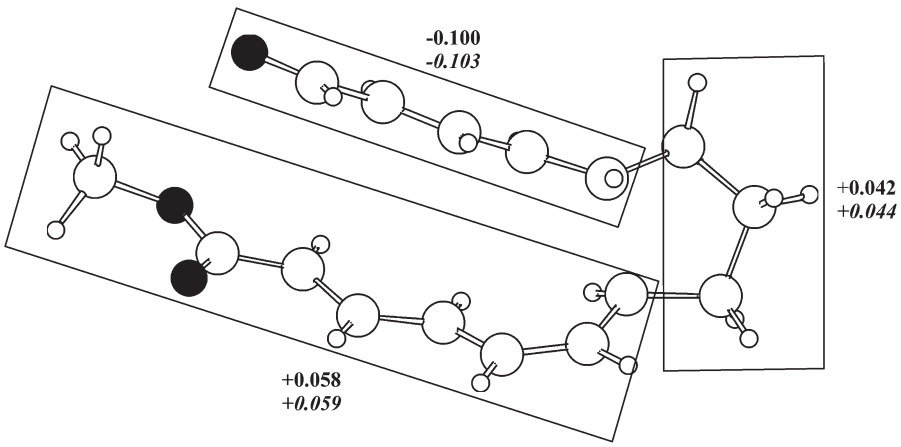

Fig. 7 Computed charge transfer for transition structure **6**. The values from both B3LYP and mPW1PW91 (in italics) are presented. Note that the positive charge is present not only in the dienophile part (lower box) of the transition structure, but also in the three methylenes that link the dienophile to the diene part.

planar carbon skeletons in order to maximize stabilization of the charge distribution in the transition structure. Secondly, the conformation about the single sp^2–sp^2 bonds in the diene and triene is fixed in **2** by its cyclic structure. Examination of a Dreiding® model of **2** however does show that there are four possible conformers arising from 180° rotations about the –(O═C)–CH– and –OOC–CH bonds in **2**. They correspond to **6** and its three-conformeric transition structures **9–11**, all of which are depicted in Fig. 8. The results of an IRC calculation for **9** (see ESI†) paralleled that reported above for transition structure **6**.

A second model system, which maintained the lactone ring, was also considered. It contained the lactone in the parent system **2** without the two alkyl and two hydroxyl groups. This conformer is not meant necessarily to be the very one that is formed when encapsulated in the enzyme that is catalyzing the DA reaction, since as mentioned above other conformers of the lactone are possible. In Fig. 9 is depicted a transition structure that was located for this model system. The results of an IRC performed on this transition structure showed that **12** links the monocyclic reactant **13** with tricyclic **14** (see Fig. 10).

Plots of the IRC points *vs.* the bond distances of the two forming sigma bonds (**A**) and the energies corresponding to these points (**B**) are presented in Fig. 11. It is seen that the process of forming the bonds qualitatively resembles the graphical representation of the IRC of the smaller model discussed above (see Fig. 5 and 6). The major difference is that now the formation of the second sigma bond proceeds more rapidly than in the "untethered" model system **12**.

While the above calculations establish that a DA pathway is certainly available to the SpnF enzyme, one needs to consider if it is energetically feasible for an enzymatic reaction. The activation energy (ΔE_a) is a crucial quantity in this respect. Although

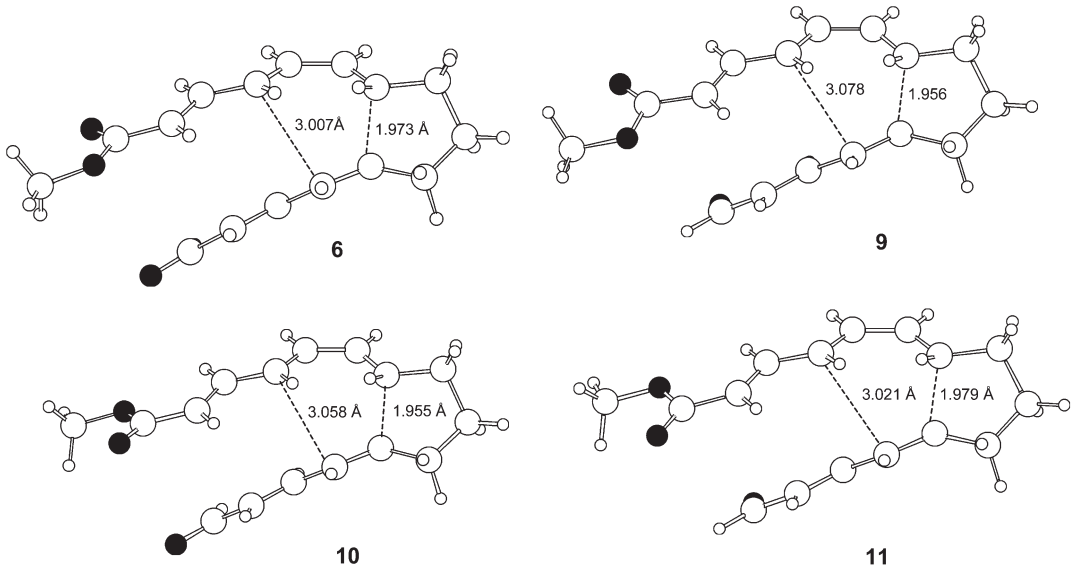

Fig. 8 Four conformeric mPW1PW91/6-311+G(2d,p) transition structures. The oxygens are black.

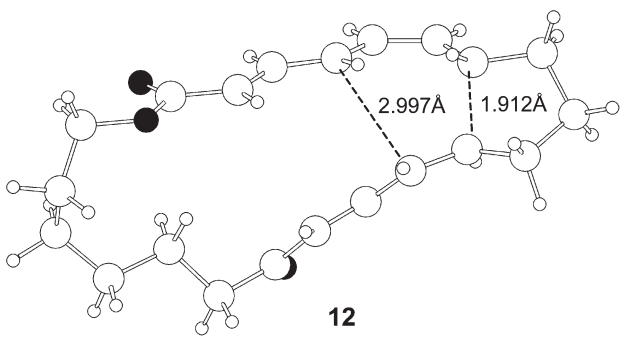

Fig. 9 A model B3LYP/6-31G* transition structure of the macrocycle **2**.

DA reactions between non-polar reactants have rather high activation energies, it has been shown that those with polar adducts are significantly lower.[20,21,25] The DFT calculated activation energies of the conversion of the model reaction **7** to **6**, for the two functionals employed, predict a ΔE_a of about 20 kcal mol^{-1}. However it known that the DFT method overestimates experimental DA activation energies, while those evaluated with the *ab initio* MP2 method are underestimated to about the same degree.[27] The calculated MP2 ΔE_a for this reaction is 10 kcal mol^{-1}. Hence for the model system we estimate it to be about 15 kcal mol^{-1}. The possibility of additional stabilization of the highly polar transition structure (large CT) by the enzyme might lower the activation energy to a value for which the reaction could easily be catalyzed enzymatically. All three methods of calculation predict the overall conversion of **7** to **8** to be exothermic and therefore energetically favored.

Computational details

All calculations were performed with GAUSSIAN 09.[28] Geometries were optimized and frequencies calculated using both the B3LYP/6-31G*[29,30] and mPW1PW91/6-311+G(2d,p)[31] for the density functional calculations (DFT), except for structure **12** where only B3LYP/6-31G* was used. In addition, *ab initio* Moller–Plesset[32] (MP2/6-31G*) geometry optimizations for **6–8** were also performed. All reported energies include zero-point energy corrections (unscaled). Fig. 3, 4 and 8 depict the mPW1PW91/6-311+G(2d,p) optimized geometries, and Fig. 9 and 10 the B3LYP/6-31G* optimized geometries. The B3LYP/6-31G* method was used for all IRC calculations.[22,23] The Gaussian program default value for the stepsize in these calculations was used. While the B3LYP method is known to perform reasonably well in the prediction of geometries and computed activation energies, it gives very poor energies of reaction, especially when carbocyclic rings are formed during the course of the reaction, as pointed out by Matsuda *et al.*[33] He found that single point energy calculations [(mPW1PW91/6-31+G(d,p)// B3LYP/6-31+G(d,p)] provide excellent estimates of overall reaction energies. Here, except for **12–14**, the structures were small enough that we were able to carry out geometry optimizations with the mPW1PW91/6-311+G(2d,p) method. All DFT energies reported in the manuscript are the results of this method except for **12–14**, where single point mPW1PW91/6-311+G(2d,p)// B3LYP/6-31G* are reported. Charge transfer in the transition structure **6** was computed from the natural population analysis.[34,35]

Conclusions

The results of calculations based on two model systems of the proposed reactant for a DA reaction in spinosyn A indicate that for both the acyclic and cyclic models it is possible to locate transition structures appropriate for asynchronous, concerted reactions of the diene and dienophile present in the analyzed systems. Energy considerations also point to the likelihood that this DA reaction can undergo catalysis by an enzyme. Based on these computational results it is concluded that the cyclase SpnF catalyzes a concerted, highly asynchronous DA reaction, as

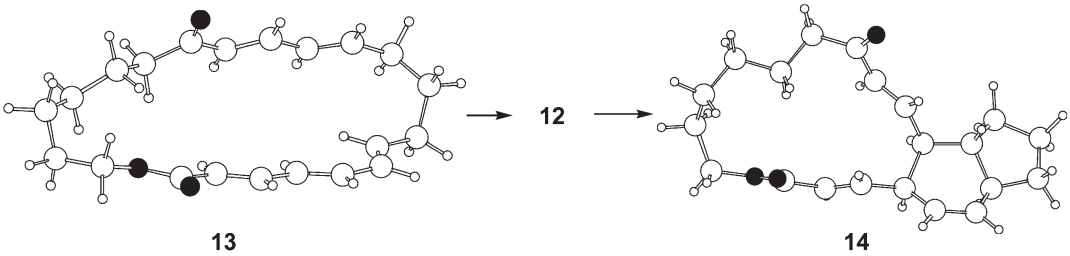

Fig. 10 Reactant and product for the lactone model system as generated by B3LYP/6-31G*.

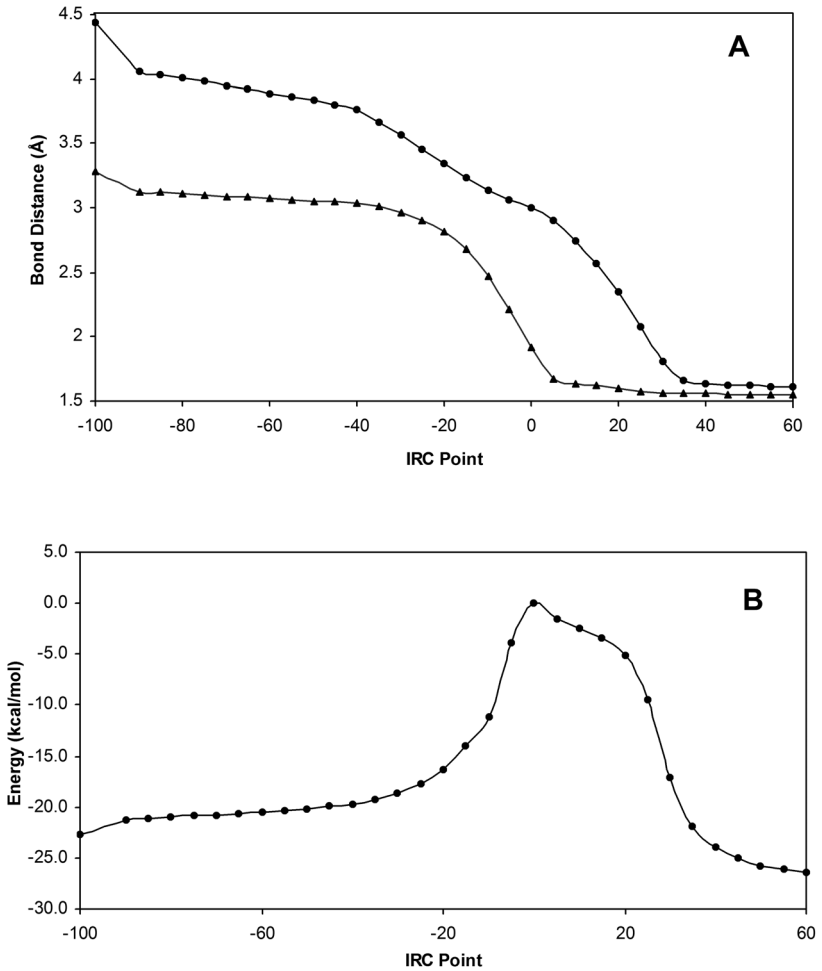

Fig. 11 The IRC plots of transition structure **12**. Points −100 and 60 represent the reactant and product respectively. **A**. Progress of the two forming sigma bonds during the course of the [4 + 2] cycloaddition. The initially formed bond is shown with ▲ and the second bond with ●. **B**. Changes in energy in relation to the energy of the transition structure.

proposed by Kim *et al.*,[2] rather than a stepwise reaction with a zwitterionic intermediate. The results of the numerical analysis presented here strongly support the conclusion that SpnF is the first stand-alone "Diels–Alderase" to be identified.[36]

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35 A. E. Reed, L. A. Curtiss and F. Weinhold, *Chem. Rev.*, 1988, **88**, 899–926.
36 There appears to be some disagreement over the definition of a Diels–Alder reaction. A referee has commented, "a Diels–Alder reaction is defined as a reaction that yields a [4 + 2] cycloadduct regardless of mechanism." However, Kelly (ref. 12) states, "The hallmark of Diels–Alder [4 + 2] cycloadditions is that they are concerted — they proceed without forming any transient intermediates en route to the final product." We, as has Kim (ref. 2), have adopted the latter definition.