Biosynthesis

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Biosynthetic Mechanism of Lanosterol: Cyclization**

Nanhao Chen, Shenglong Wang, Lidia Smentek, B. Andes Hess, Jr., and Ruibo Wu**

Abstract: *The remarkable cyclization mechanism of the formation of the 6-6-6-5 tetracyclic lanosterol (a key triterpenoid intermediate in the biosynthesis of cholesterol) from the acyclic 2,3-oxidosqualene catalyzed by oxidosqualene cyclase (OSC) has stimulated the interest of chemists and biologists for over a half century. Herein, the elaborate, state-of-the-art two-dimensional (2D) QM/MM MD simulations have clearly shown that the cyclization of the A–C rings involves a nearly concerted, but highly asynchronous cyclization, to yield a stable intermediate with "6-6-5" rings followed by the ring expansion of the C-ring concomitant with the formation of the D-ring to yield the "6-6-6-5" protosterol cation. The calculated reaction barrier of the rate-limiting step (≈22 kcal mol^{-1}) is comparable to the experimental kinetic results. Furthermore all previous experimental mutagenic evidence is highly consistent with the identified reaction mechanism.*

The biogenetic mechanism of the formation of cholesterol is one of the most complicated biosyntheses, which has been studied for over a half century. However many of the details of the mechanism still remain controversial.[1] As early as 1966, the four-ring lanosterol was demonstrated to be a key intermediate in the cholesterol biosynthesis, which is cyclized by 2,3-oxidosqualene cyclase (OSC) from the acyclic 2,3-oxidosqualene (Figure 1 a). Furthermore, the cyclization by OSCs was the first diversifying step in triterpenoid biosynthesis in animals and plants.[2] Therefore, this efficient and fascinating cyclization reaction has over the past 50 years attracted the attention of organic chemists, synthetic biologists, and theoretical chemists.[3] Renewed interest in the study of this mechanism[4] was generated by Hess's finding[5] in 2002 that the "anti-Markovnikov" ring expansion of the tertiary 6-6-5 intermediate carbocation to the secondary 6-6-6 intermediate could be avoided by a concerted ring expansion/

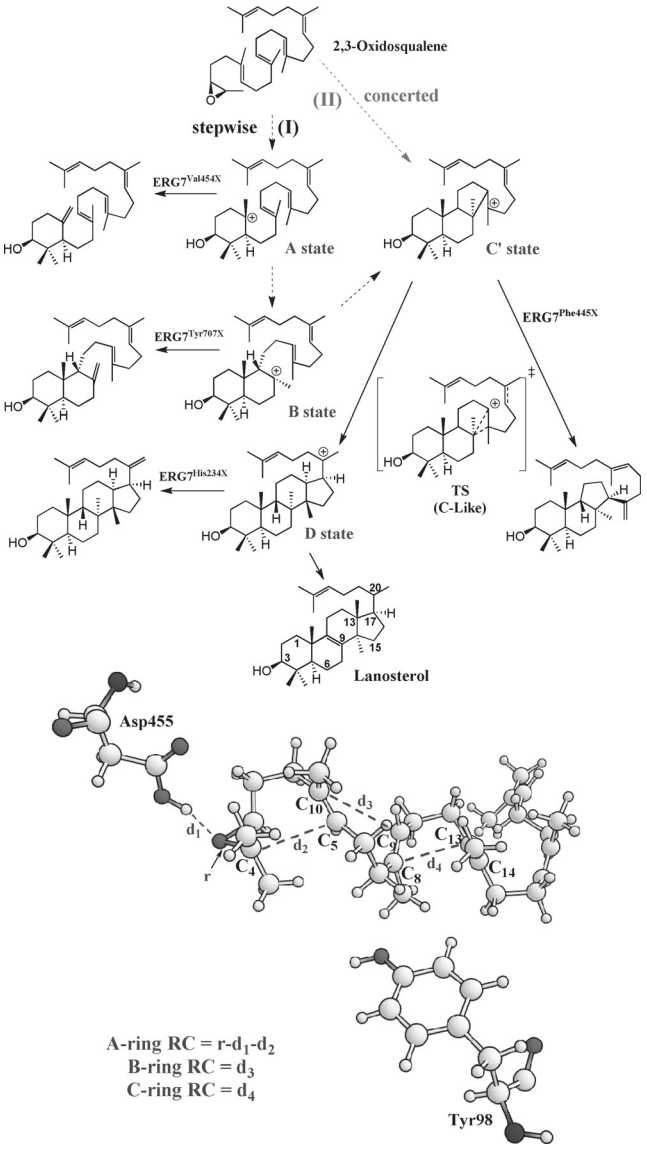

Figure 1. a) The mainstream cyclization hypotheses of OSC and some key mutations. b) The definition of the reaction coordinates and the QM subsystem.

ring closure to go directly from the 6-6-5 intermediate to the 6-6-6-5 protosterol carbocation as well as by Ruf's determination in 2004 of the X-ray crystal structure of the human OSC.[6] In addition, OSC has attracted much attention in recent years as a novel antihypolipidemic target.[7] The most likely cyclization process is shown in Figure 1 a.[3f,g,4,5] So far, a one-step cyclization has been excluded. It has been shown that a stable C′ state (6-6-5 ring) likely exists and is subsequently converted to the D state (6-6-6-5 ring) with a low barrier (≈6 kcal mol^{-1}) on the basis of our previous

[*] N. Chen, Prof. R. Wu
School of Pharmaceutical Science, Sun Yet-sen University
Guangzhou, 510006 (China)
E-mail: wurb3@mail.sysu.edu.cn
Prof. Dr. L. Smentek, Prof. B. A. Hess, Jr.
Department of Chemistry, Vanderbilt University
Nashville, TN37235 (USA)
E-mail: b.andes.hess@vanderbilt.edu
Dr. S. Wang
Information Technology Services, New York University
New York, NY10003 (USA)

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quantum mechanics/molecular mechanics molecular dynamics (QM/MM MD) simulations on the complete enzyme system,[4e] which is also consistent with Hess's calculations[4b,d,5] in the gas phase. However, the cyclization of the A, B, and C rings of the protosterol cation is more complicated and still remains controversial. So far, the A and B state intermediates have been proposed to exist indirectly based on several mutant experiments (Figure 1 a).[8] The A state was also predicted to be stable by Eriksson's QM/MM calculation.[9] In striking contrast, Hess[4d] pointed out that both the A and B states are unstable from a first principles calculation in the gas phase.

In the current study, as illustrated in Figure 2 a, the free energy surface along the A-ring RC (r-d1-d2) and B-ring RC (d3) (see Figure 1 b and Experimental Section) and atomic distance evolvement (Figures S3 and S4) confirmed that the ring-opening of epoxide and the cyclization of the A ring is concerted but asynchronous, with a barrier of 21.8 kcal mol^{-1}. As seen from the reaction pathway as shown by a white, dashed line in Figure 2 a, there is an asynchronous cyclization of the A and B rings. Furthermore, it is kinetically very facile (< 1 kcal mol^{-1} barrier) and thermodynamically favorable (≈ 10 kcal mol^{-1} exothermicity) from the A to the B state. Thus the A state is metastable.

The B state seems stable in view of the free energy surface. However, it is also metastable on the basis of a further cluster analysis of all structures from the QM/MM MD simulations. As shown in Figure 2 b, it should be divided into two clusters which correspond to the B and C′ states (Figure 1 a). The d4 is so fluxional during the simulations that about 46 % of the sampling points are located in the C′ state, whereas only 22.3 % represent the B state. So the next query is: Why are both the A and B states metastable?

Fortunately, the free energy surface from A to C′ state makes it clear, as shown in Figure 2 c. Obviously, the A state is located in a high-energy region and easily falls to a lower-energy region with a very small barrier. With regard to the B state, there is a long and narrow basin that spans from B to C′ states without any distinct barrier and energy difference between them; thus, the cyclization reaction from B to C′ state is essentially reversible. Moreover, the reaction would not likely stop in this free energy basin, because it would quickly move forward due to the next very facile cyclization from C′ to the very stable D state as predicted in our previous study.[4e]

Therefore, the overall cyclization free-energy profile can be drawn as in Figure 3. This cyclization in nature is a carbocation migration (see details in Table S1) and is

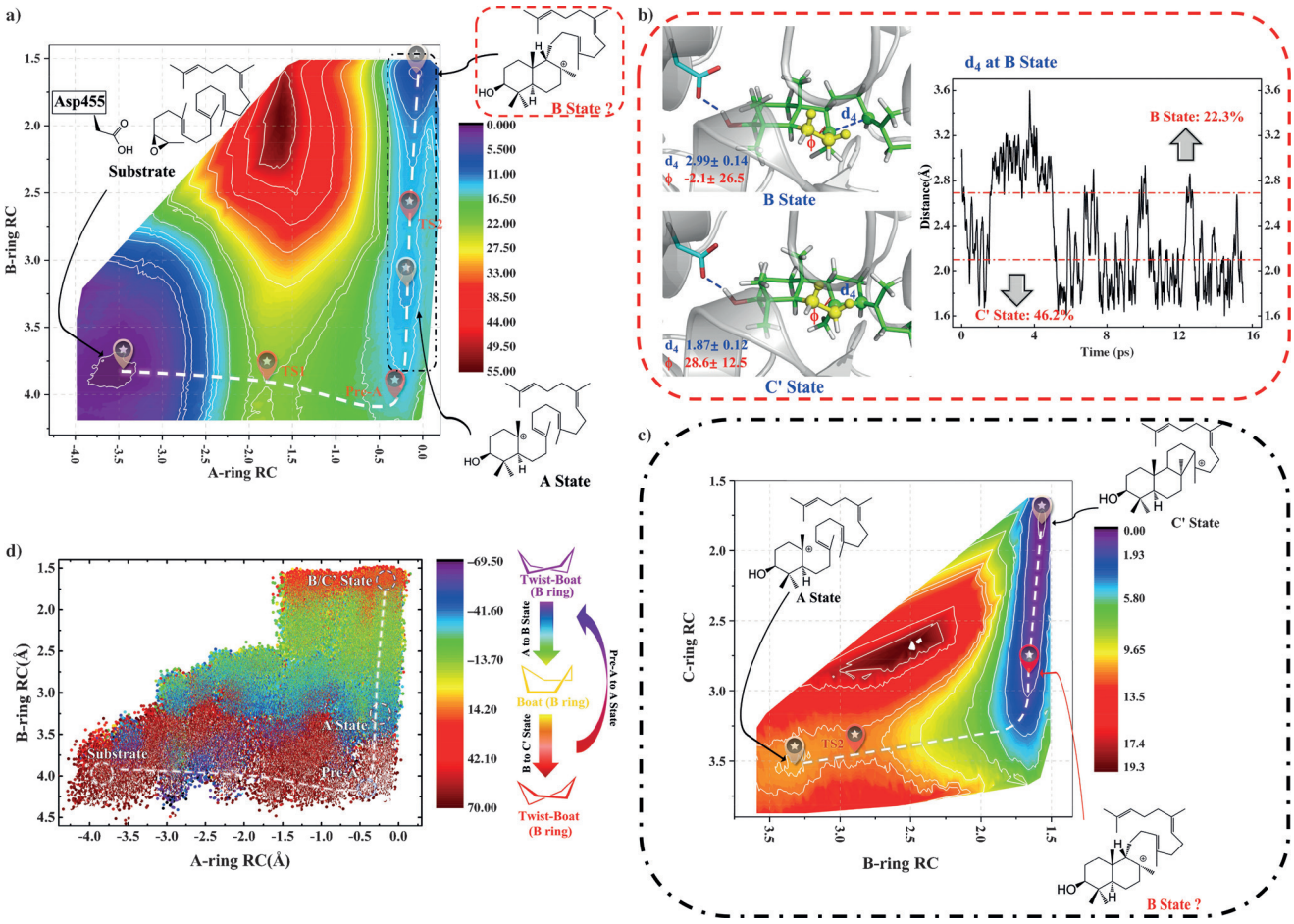

Figure 2. a) The free-energy surface (kcal mol^{-1}) along the RCs [Å] from substrate to B state. b) The distance of C-ring atoms (d_4) during the sampling of B state and two represented structures in this sampling. c) The free-energy surface from A to C′ state. d) The dihedral degree diagram from substrate to B state.

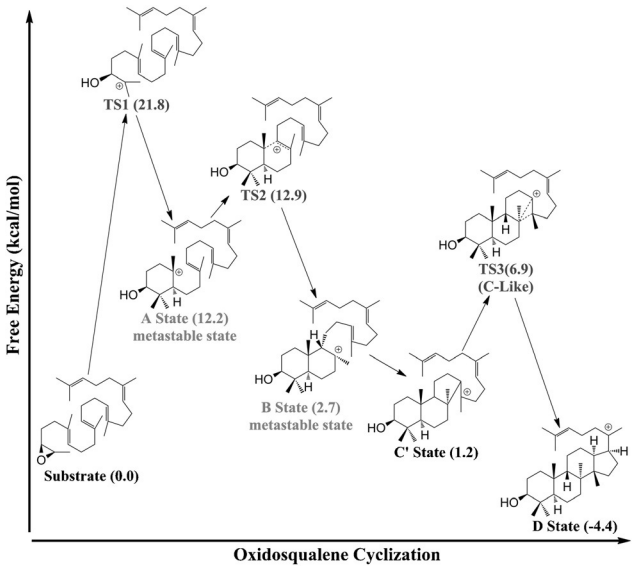

Figure 3. The whole cyclization of the oxidosqualene. The metastable states are highlighted in red and the transition states are colored in blue. The free-energy data are marked in parentheses.

exergonic (≈ 4.4 kcal mol^{-1}). The rate-limiting step is predicted to have a ca. 22 kcal mol^{-1} barrier for the concerted formation of the A ring and opening of the epoxide ring. To the best of our knowledge, the direct experimental kinetic data of the human OSC has not yet been determined, though it is comparable in value to that reported for bovine and rat lanosterol synthases,[10] 23.3 and 24.3 kcal mol^{-1}, respectively. With regard to Eriksson's work with a static QM/MM method,[9] our metastable A state is quite different from his very stable A state (Figure S5), and his strongly endothermic ($+19$ kcal mol^{-1}) for the overall cyclization is in direct contrast with our results (see below). It is interesting to note that the transition structure of this rate-determining step (TS1 in Figure 3) is very similar to the transition structure located by Hess[4d] for the gas phase, concerted cyclization of the A–C rings (Figure S6).

The relative free energy for each state is much different from Hess's[4d] results in the gas phase calculation level in which he employed a truncated model that included neither the epoxide nor the enzyme environment around the substrate. However, he found an asynchronous cyclization of the A, B, and C' rings and that the order of their relative energies is consistent with results presented here. In fact Hess's energy for the cyclization of the A–C rings is not unexpected, because it is in good agreement with Matusda's extensive computational study of squalene and squalene oxide cyclizations.[11] Wendt, Poralla, and Schulz pointed out in 1997 that the very similar cyclization of squalene to hopene should release about 48 kcal mol^{-1}, which far exceeds the usual protein stabilization energy of about 12 kcal mol^{-1} and is an order of magnitude different from our OSC system.[12] Our results here suggest that the actual energy released during the cyclization is only about 5 kcal mol^{-1}, which is not sufficient to denature the protein. What is the role of the enzyme in "moderating" this reaction by an order of magnitude? If one considers that the substrate is essentially a "key" and the enzyme is a "lock", then the cyclization will create a very different new "key", which would likely mean that a significant amount of the energy released in the gas phase is not directly released but rather used in accommodating the new "key" in the old "lock". As suggested by Wendt, Poralla, and Schulz this resulting "pent-up" energy would then be available to return the cyclized product to the membrane. Furthermore, it has been suggested that the binding affinity of the substrate analogue is better than that of the protosterol cation, which would also imply a less exoergic cyclization from substrate to protosterol cation.[13]

One key dihedral angle (see Φ in Figures 2b and S3) is very flexible from the substrate to C′ state (Figure 2d). It rapidly decreases to about $-35°$ from the pre-A state (as noted in Figure 2d) to the A state, recovers to near zero in the B state ($\Phi = -2.1 \pm 27°$), and again becomes somewhat positive in the C′ state ($\Phi = 27 \pm 10°$) (Figure S7). This readily changing dihedral angle is a result of occurring conformational changes, which result from a twist-boat conformation (A state) to a boat conformation (B state) and then again to a twist-boat conformation (C′ state) for the six-membered B ring. We note that in the earlier gas-phase calculations of Hess[4d] this dihedral angle stays positive throughout the cyclization (Φ remains about 64° for the reactant, the transition structure of the concerted reaction and cyclization of the A ring, but then decreases to 32° when the B ring is formed and remains essentially constant to the C′ state). These conformational changes convince us that it is possible that most of the exothermicity of the cyclization likely promotes local conformational rearrangements of the protein active site to fit each reaction intermediate state (new "key") with a boat-conformation twist, which leads to optimal binding modes. It raises another question of which and how these residues in the active site stabilize or destabilize the metastable intermediates.

Herein, similar QM/MM MD simulations on mutant models were also performed around the A and pre-A states and the lifetimes during the MD simulations for the pre-A and A states are summarized in Table 1. Obviously, F444 is one of the most important residues in stabilizing the metastable A state, because a F444A mutation might remove the barrier to the formation of the B state as well as

Table 1: The life time (fs) of the pre-A and A state, and the ratio of B/C′ state after 8 ps of B state formation in different QM sites simulations.

QM area (QM3 = QM1 + residue)	Pre-A	A state	B/C′ ratio [%]
None	400	1935	49
F444	460	**6053**	40
F444A	582	–	**28**
V453	400	323	52
V453A	**6445**	**9995**	47
Y503	420	822	41
Y503A	550	**5318**	37
W581	**2119**	–	**25**
W581A	764	–	52
Y704	500	1552	36
Y704A	1890	**90**	**66**
Y98	390	4247	42
Y98A	**4110**	3792	**29**

the C′ state, which would be consistent with the mutant experimental results that F445T in ERG7 changes the main product to a 6-6-5 ring state.[8c] Further ESP charge analyses show that the cyclization is a carbocation migration (see detailed discussion in Table S1). As shown in Table S2, F444 could delocalize the positive charge of the A ring (C1–C5, C10) to bring about additional stability. In contrast, the presence of V453 brings a higher positive charge to the A ring, and thus the V453A mutation would greatly extend the lifetime of the pre-A and A state as shown in Table 1, which is again quite consistent with mutant experiments in ERG7 in that about 5% of achilleol A by-product forms via V454A (namely V453 herein).[8a] Y503 might also contribute to the metastability of the A state. Compared to V454A in ERG7, Y510A (Y503 in human OSC) in ERG7 also produces about 27% of achilleol A as has been shown by previous experimental studies.[14] Moreover, we propose one novel residue, W581, which can influence the proton transfer after cyclization[15] and may also stabilize the pre-A state and promote the cyclization to C′ state. When Y704A was studied, not only the lifetime of the A state becomes very short, but the total charge of the B ring is delocalized as well. As a result, the positive charge on C10 is less in comparison with the wild type, thus the B state is stabilized; the B state would not so easily convert to the C′ state as the lifetime ratio of B/C′ state is larger than for other mutants (66%, Table 1), which is in agreement with the results of the mutant experiment Y707A in ERG7 (Y704 in human OSC) that produces a 6–6 ring by-product.[8d] Another key residue, Y98, which is proposed to be the B-boat stimulator, would promote the A state formation and stabilize the B state, whereas Y98A would propel the cyclization to achieve the C′ state (B/C′ = 29%).[16]

In summary, our simulations have characterized the cyclization reaction of squalene oxide catalyzed by OSC at the highest computational level carried out to date. The conversion of the substrate (the acyclic squalene oxide) to the C′ state is near-concerted, but asynchronous, with two metastable states (the A and B states) and a reaction barrier of about 22 kcal mol^{-1} for the rate-limiting step. Further mutant analyses elucidated the important catalytic roles of several key residues in stabilizing or destabilizing the metastable A state. Our results not only elucidate the cyclization mechanism but also show it to be consistent with most experimental kinetic data as well as mutagenesis experiments. Our hope is that these results will end the dispute lasting for over half a century. Moreover, it may provide a guide for the design of new enzymatic activity in enzyme engineering to produce diverse fused multiring triterpenes.

Experimental Section

The enzyme–substrate complex was built based on our previous calculation and the same theoretical method (M062X/6-31G*:AMBER99SB) was employed.[4e] The detailed choices of reaction coordinates (RC) and QM regions were discussed in Figure 1b and Figures S1, S2, and S8. The 2D umbrella sampling was applied to sample the variables and WHAM for the 2D free-energy surface calculations. Additional computational details are given in the Supporting Information.

Keywords: biosynthesis · cyclization · lanosterol · oxidosqualane cyclase · QM/MM

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[1] a) K. U. Wendt, G. E. Schulz, E. J. Corey, D. R. Liu, *Angew. Chem. Int. Ed.* **2000**, *39*, 2812–2833; *Angew. Chem.* **2000**, *112*, 2930–2952; b) T. K. Wu, C. H. Chang, Y. T. Liu, T. T. Wang, *Chem. Rec.* **2008**, *8*, 302–325.
[2] S. Sawai, K. Saito, *Front. Plant Sci.* **2011**, 2.
[3] a) E. J. Corey, W. E. Russey, *J. Am. Chem. Soc.* **1966**, *88*, 4751–4752; b) E. E. Van Tamelen, J. Willet, M. Schwartz, R. Nadeau, *J. Am. Chem. Soc.* **1966**, *88*, 5937–5938; c) E. E. Van Tamelen, J. D. Willett, R. B. Clayton, K. E. Lord, *J. Am. Chem. Soc.* **1966**, *88*, 4752–4754; d) E. E. Van Tamelen, K. B. Sharpless, R. P. Hanzlik, R. B. Clayton, A. L. Burlingame, P. C. Wszolek, *J. Am. Chem. Soc.* **1967**, *89*, 7150–7151; e) J. D. Willett, K. B. Sharpless, K. E. Lord, E. E. van Tamelen, R. B. Clayton, *J. Biol. Chem.* **1967**, *242*, 4182–4191; f) C. Jenson, W. L. Jorgensen, *J. Am. Chem. Soc.* **1997**, *119*, 10846–10854; g) D. Gao, Y.-K. Pan, K. Byun, J. Gao, *J. Am. Chem. Soc.* **1998**, *120*, 4045–4046.
[4] a) T. Hoshino, T. Sato, *Chem. Commun.* **2002**, 291–301; b) B. A. Hess, Jr., *Org. Lett.* **2003**, *5*, 165–167; c) W. D. Nes, *Chem. Rev.* **2011**, *111*, 6423–6451; d) B. A. Hess, L. Smentek, *Angew. Chem. Int. Ed.* **2013**, *52*, 11029–11033; *Angew. Chem.* **2013**, *125*, 11235–11239; e) N. H. Chen, J. W. Zhou, J. B. Li, J. Xu, R. B. Wu, *J. Chem. Theory Comput.* **2014**, *10*, 1109–1120; f) D. J. Tantillo, *Nat. Prod. Rep.* **2011**, *28*, 1035–1053.
[5] B. A. Hess, Jr., *J. Am. Chem. Soc.* **2002**, *124*, 10286–10287.
[6] R. Thoma, T. Schulz-Gasch, B. D'Arcy, J. Benz, J. Aebi, H. Dehmlow, M. Hennig, M. Stihle, A. Ruf, *Nature* **2004**, *432*, 118–122.
[7] a) S. Lange, M. Keller, C. Muller, S. Oliaro-Bosso, G. Balliano, F. Bracher, *Eur. J. Med. Chem.* **2013**, *63*, 758–764; b) N. S. H. N. Moorthy, N. M. F. S. A. Cerqueira, M. J. Ramos, P. A. Fernandes, *RSC Adv.* **2013**, *3*, 23409–23422; c) D. Staedler, C. Chapuis-Bernasconi, H. Dehmlow, H. Fischer, L. Juillerat-Jeanneret, J. D. Aebi, *J. Med. Chem.* **2012**, *55*, 4990–5002.
[8] a) B. M. Joubert, L. Hua, S. P. T. Matsuda, *Org. Lett.* **2000**, *2*, 339–341; b) T. K. Wu, Y. T. Liu, C. H. Chang, M. T. Yu, H. J. Wang, *J. Am. Chem. Soc.* **2006**, *128*, 6414–6419; c) T. K. Wu, Y. T. Liu, F. H. Chiu, C. H. Chang, *Org. Lett.* **2006**, *8*, 4691–4694; d) T. K. Wu, T. T. Wang, C. H. Chang, Y. T. Liu, W. S. Shie, *Org. Lett.* **2008**, *10*, 4959–4962.
[9] B. X. Tian, L. A. Eriksson, *J. Phys. Chem. B* **2012**, *116*, 13857–13862.
[10] a) W. R. Moore, G. L. Schatzman, *J. Biol. Chem.* **1992**, *267*, 22003–22006; b) T. K. Wu, C. Y. Huang, C. Y. Ko, C. H. Chang, Y. J. Chen, H. K. Liao, *Arch. Biochem. Biophys.* **2004**, *421*, 42–53.
[11] S. P. T. Matsuda, W. K. Wilson, Q. B. Xiong, *Org. Biomol. Chem.* **2006**, *4*, 530–543.
[12] K. U. Wendt, K. Poralla, G. E. Schulz, *Science* **1997**, *277*, 1811–1815.
[13] E. J. Corey, D. C. Daley, H. M. Cheng, *Tetrahedron Lett.* **1996**, *37*, 3287–3290.
[14] T. K. Wu, C. H. Chang, *ChemBioChem* **2004**, *5*, 1712–1715.
[15] C. H. Chang, H. Y. Wen, W. S. Shie, C. T. Lu, M. E. Li, Y. T. Liu, W. H. Li, T. K. Wu, *Org. Biomol. Chem.* **2013**, *11*, 4214–4219.
[16] T. K. Wu, W. H. Li, C. H. Chang, H. Y. Wen, Y. T. Liu, Y. C. Chang, *Eur. J. Org. Chem.* **2009**, 5731–5737.

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