Published in final edited form as:

Medchemcomm. 2012 January 1; 3: 960–966. doi:10.1039/C2MD00305H.

Engineering the synthetic potential of β -lactam synthetase and the importance of catalytic loop dynamics†

Jason W. Labonte^a, Fumitaka Kudo^a, Michael F. Freeman^a, Mary L. Raber^a, and Craig A. Townsend^a

Craig A. Townsend: ctownsend@jhu.edu

^aDepartment of Chemistry, Johns Hopkins University, 3400 N. Charles St., Baltimore, MD, USA.

Fax: 410 516 8420; Tel: 410 516 7444

Abstract

The 2-azetidinone ring of the Class A and D β -lactamase inhibitor clavulanic acid (**1**) is synthesized by the ATP-utilizing enzyme β -lactam synthetase (β LS). A hydroxyethyl group attached to C-6 of **1** in the (*S*) configuration markedly enhances the efficacy of this compound against Class C β -lactamases. Guided by a series of X-ray structures of β LS, we have engineered this enzyme to act upon a methylated substrate analogue to give selectively the (3*S*)-methyl β -lactam core, which, upon closure of the second ring of the bicyclic system of **1**, would lead to the (6*S*)-methylated clavulanic acid derivative.

Introduction

While only a weak antibiotic, clavulanic acid (**1**), a member of the β -lactam family of natural products, is a potent inhibitor of most Class A and D β -lactamases, serine proteases that prefer penams, but is inactive against both Class B metallo- β -lactamases and Class C cephalosporinases.¹

The (8*R*)-hydroxyethyl side chain found naturally on the α face of the related β -lactam thienamycin (**2**) and, for example, in the synthetic 1- β -methyl carbapenem antibiotic

†Electronic Supplementary Information (ESI) available: detailed synthetic procedures, description of kinetic assays and molecular modeling. See DOI: 10.1039/b000000x/

Correspondence to: Craig A. Townsend, ctownsend@jhu.edu.

meropenem (**3**) is important for antibiosis as well as resistance to β -lactamases. It has been proposed that this moiety restricts water access to the active site of the β -lactamase after acylation to hinder hydrolysis and regeneration of the active enzyme.^{2,3} Alternatively, it has been suggested that the hydroxyethyl forces a conformational change, placing the carbonyl of the covalent intermediate outside of the oxyanion hole, blocking hydrolysis.^{4,5} In keeping with either rationale, synthetic modification of clavulanic acid to attach a hydroxyethyl group at C-6 confers improved activity against Class C cephalosporinases. Interestingly, attachment of this group on the β face of β -lactam **4** affords significantly greater inhibitory potency than the α face.⁶ In this paper we describe experiments to rationally engineer a clavulanic acid biosynthetic enzyme to achieve synthesis of the β -lactam ring bearing a simple alkyl substituent stereoselectively at this site.

The 2-azetidinone ring of **1** is formed early in the biosynthesis by the enzyme β -lactam synthetase (β LS) in the presence of ATP (Scheme 1).⁷⁻⁹ β LS is clearly descended from the highly conserved and widely distributed superfamily of Class B asparagine synthetases (AS-B) but has completely lost its ability to convert the primary amino acid L-aspartate to L-asparagine. Comparing the X-ray structure of *E. coli* AS-B and that of β LS reveals extensive remodeling of the active site pocket to accommodate a larger substrate, *N*²-(2'-carboxyethyl)-L-arginine (CEA, **5**), yet retaining residues critical for ATP binding and orientation, and activation. The reaction proceeds by acyl adenylation of this open-chain β -amino acid, followed by closure to the four-membered lactam intermediate deoxyguanidino-proclavaminic acid (DGPC, **6**) mediated by a Tyr-Glu catalytic dyad.¹⁰

A series of five X-ray structures of β LS with substrates, products, and non-reactive cofactor or substrate analogs was obtained.^{11, 12} This sequence of images has provided rare but lucid detail of the catalytic cycle as substrates bind, react, and yield products while surrounding residues move in a coordinated fashion within the active site as the synthetic events proceed (Fig. 1).

While the overall biosynthetic pathways to the known β -lactam classes are strikingly dissimilar, one point of mechanistic overlap is with carbapenam synthetase (CPS, Scheme 2), an enzyme involved in the biosynthesis of the simplest carbapenem β -lactam antibiotic, carbapenam-3-carboxylic acid (**7**). CPS catalyzes an analogous reaction to β LS, closing (2*S*, 5*S*)-carboxymethylproline (CMPr, **8**) to the carbapenam **9** (Scheme 2). Primary sequence alignment and X-ray structures show that CPS and β LS share residues important for substrate and ATP binding, activation, and catalysis, despite modest overall sequence identity (22%) and distinctly shaped active site cavities.¹³

The static images of the reaction cycle afforded by the X-ray structures have been coupled to the dynamics of the complex two-step transformation by extensive kinetic and computational studies.^{10, 14, 15} To summarize these findings, substrate pre-organization, adenylation, and placement of a catalytic dyad and a critical lysine, which contributes to both ATP-binding and activation of β -lactam closure, adroitly facilitate the enthalpically uphill process of β -lactam formation as illustrated in Scheme 1. So successful is the orchestration of catalytic effects that the overall rate of reaction is wholly or in part limited by a conformational change in the respective proteins to allow product release.¹⁴ A disordered loop region in β LS (residues 444–453, shared by homolog carbapenam synthetase)¹⁶ becomes ordered in one monomer each of the adenylated intermediate and product structures (Fig. 1C and D) to enclose the active site as chemistry ensues. It seems logical that relaxation back to the disordered state then leads to full product release in keeping with the kinetic observations.

These structural and kinetic insights have been exploited to engineer β LS to bind, adenylate, and cyclize a slightly larger substrate from stereospecific introduction of a short, neutral, alkyl chain on the substrate CEA (**5**). It is known that similarly placed side chains in carbapenem substrates can be regio- and stereospecifically hydroxylated enzymatically to give the (8*R*)-hydroxyl group present, for example, in thienamycin (**2**).¹⁷ To accommodate a larger substrate, the V446A and V446G mutants of β LS were selected to evaluate the diastereomeric preference of these variants for a (2'*S*)- or (2'*R*)-methyl group in *N*²-(2'-carboxypropyl)-L-arginine (2'-Me-CEA diastereomers, **10**, Scheme 3) in the two-step β -lactam-forming reaction. A marked preference for the (2'*S*)-methyl substitution was observed. Consideration of these findings using molecular modeling of the β LS active site is described along with the results of altering His447, which is situated in the active site on the opposite side of these substrates on the catalytic loop.

Results and discussion

From the series of β LS crystal structures, it can be seen that binding to the substrate, CEA (**5**), is facilitated by a backbone hydrogen bond from Gly349 to the carboxylate of the arginine moiety and by interactions with the guanidino group from Glu382, Asp373, and a water molecule (Fig. 1B). When the catalytic loop is open, the carboxyethyl portion of the substrate appears free to sample the space around the active site (as can be seen in subunit B of PDB ID#1JGT); however, when the loop is closed and CEA has been adenylated, as evidenced by the CMA-AMP structure (Fig. 1C), movement of the carboxyethyl group is limited. Flexibility of the carboxyethyl group is also supported by higher B factors in the X-ray structures for this moiety compared to the carboxylate or guanidino groups of the substrate. These observations open the possibility for modifications to the carboxyethyl portion of the molecule — such as at the 2' position — without substantially affecting substrate binding. (It should be noted that the 2' position of CEA (**5**) becomes C-3' in the β LS product DGPC (**6**) and C-6 in the final biosynthetic product. See Scheme 1.)

The synthetic potential of wild-type (WT) β LS had been preliminarily probed with a *ca.* 1:1 mixture of 2'-Me-CEA diastereomers **10**, which, when incubated with β LS in a fixed-time assay, gave a similar mixture of stereochemically undefined 3'-methylated DGPC isomers.¹⁸ To examine more rigorously the synthetic capability of β LS and mutants selected on the basis of protein structure and mechanism, substrate CEA (**5**) and analogs bearing 2'-methyl and 2'-ethyl substituents were prepared stereospecifically as outlined in Scheme 3. Saponification of the commercially-available β -hydroxyisobutyric acid esters gave **11a** and **11b**, which were coupled to doubly protected ornithine **12**. The optically pure diastereomers **13** were separately cyclized in a modified Mitsunobu reaction¹⁹ and deprotected to give **14a** and **14b**. Aminoiminomethanesulfonic acid was utilized as an electrophilic guanidino source²⁰ to give β LS product derivatives 3'-methyldeoxyguanidinoproclavaminic acids (3'-Me-DGPC, **15**), which in turn were purified by HPLC and hydrolyzed to the desired stereopure CEA derivatives.

Recombinant β LS was prepared from a partially codon-optimized clone and purified as previously described.¹⁴ Site-specific mutants of β LS were obtained using the overlap extension method.¹⁴ Progress of the enzyme reactions was monitored by AMP release in a coupled-enzyme assay, and the data were fit by non-linear regression.¹⁴

With WT β LS (2'*S*)-Me-CEA (**10a**) showed a very small increase in K_M and a ~45-fold decrease in k_{cat} relative to natural substrate CEA (**5**); (2'*R*)-Me-CEA (**10b**) similarly showed no change in K_M and about a 30-fold decrease in k_{cat} (Table 1). These moderate kinetic changes suggested that the shape of the active site of β LS might be modified to better accommodate alternate substrates while maintaining its two-step reaction cycle. As a

first step in this direction, mutants were prepared in the active site region proximal to the carboxyethyl moiety of the bound substrates. V446A, residing on the catalytic loop, and Y326F (Fig. 1) were the first mutants chosen. With the natural substrate CEA, Y326F marginally outperformed WT β LS with a slightly higher k_{cat} value, but a higher K_M . With the alternate substrates **10**, however, the Y326F mutant functioned poorly, perhaps providing inadequate room in the active site to sustain efficient catalysis of the 2'-substituted CEA analogs. These initial experiments were not pursued further (data not shown).

Next, the β LS variant V446A with CEA (**5**) showed a ten-fold increase in K_M and only a three-fold reduction in k_{cat} . With the two 2'-Me-CEA diastereomers, however, V446A showed a marked preference for the (2'*S*) diastereomer **10a** over its (2'*R*) diastereomer (**10b**). Both compounds had similar K_M values with V446A, implying that the distinction in activity occurs principally during catalysis and not binding to the enzyme. The k_{cat} value for **10b** with V446A is about half that of WT. The kinetic parameters for **10a** in V446A, on the other hand, are almost identical to those for CEA with this mutant (Table 1). Thus, while **10a** turns over as poorly as **10b** in WT β LS, reducing the steric demand of Val446 creates a pocket on one side of the active site to permit **10a** to turn over 20 times faster than **10b** and approximately as well as the native, unsubstituted substrate, CEA.

With this stereochemical discrimination in mind, the proposed tetrahedral intermediate of the reaction was modeled using the Accelrys Discovery Studio Suite into the active site of the adenylated intermediate β LS structure (PDB ID#1MBZ, subunit A), guided by DGPC (**6**) binding information from the product structure (PDB ID#1MC1, subunit A), to provide a rationale for the diastereoselective reactivity observed above (Fig. 2). This model would suggest that both 2'-Me-CEA derivatives **10** should bind equally well to the open conformation of β LS, in agreement with their measured K_M values compared to that of CEA (**5**). When the catalytic loop is ordered, however, **10a** should still fit in the active site with its attached methyl group filling the void afforded by the replacement of Val446 with alanine. Recall that placement of the alkyl group on the β -face of the β -lactam in clavulanic acid (*cf.* **4**) was clearly favorable for inhibitory activity against Class C β -lactamases.⁶

In contrast, the optimal C-6 side chain geometry for desirable antibiotic and β -lactamase inhibitor activity in thienamycin (**2**) and related compounds would place this group on the α face of the β -lactam nucleus — that is, arising from the (2'*R*)-alkyl CEA stereochemistry. According to our model, this diastereomer, **10b**, would continue to sterically clash with the other side of the active site, particularly the region occupied by His447.

As a consequence, we prepared a H447A β LS mutant and tested it against CEA and **10**, but while this mutant did not have a strong effect on the binding of native substrate CEA (**5**), the k_{cat} was reduced by about 50-fold relative to WT (Table 1). Further against the expected outcome of this model, kinetic measurements demonstrated that H447A continued to prefer **10a** over **10b**, albeit weakly (Table 1). When the catalytic loop is closed, the X-ray structural data reveal that His447 is in close contact with Tyr326, perhaps in a favorable π -stacking arrangement (Fig. 1C and D and Fig. 3C–H). Furthermore, His447 can hydrogen bond with Thr453 (Fig. 3H). Disruption of these interactions may be the cause of this loss in catalytic efficiency by affecting the ability of the catalytic loop to close properly. This histidine is fully conserved in all known homologous β -lactam synthesizing enzymes with the exception of T β LS in tabtoxin biosynthesis, which does not contain the same catalytic loop.²¹ In the case of CPS from simple carbapenem biosynthesis, this histidine (also His447) may interact with either or both Tyr467 and Glu322, which occupy the same region as Thr453 is in β LS. In ThnM, the CPS homolog active in thienamycin biosynthesis,²² Thr453 is replaced by Ser407, which presumably would interact with His403 in that enzyme. On the

other hand, Val446 is substituted by an isoleucine in a β LS paralog²³ and in both CPS¹⁶ and ThnM and their homologs²⁴, so mutation here to another nonpolar residue must not have a great effect on the wild-type reaction, as confirmed by the behavior of the V446A mutation.

It could well be that most of the residues on the catalytic loop are involved in its structured folding (Fig. 3). We hypothesize that cleavage between the α - and β -phosphates during the adenylation step reorients the magnesium ions and coordinated phosphate oxygens (due to stereochemical inversion at phosphate α as a consequence of adenylation) such that the essential Lys443 can cap three oxygen atoms rather than two. (Compare Fig. 1A and B with Fig. 3A.) This change pulls down on the loop drawing the backbone carbonyl of Leu444 to join one of the magnesium ion coordination spheres (Fig. 3B). His447, as mentioned above, may π -stack with Tyr326 (Fig. 3C). Continuing downstream along the loop, Glu448 hydrogen-bonds with the amide nitrogen of Gly445 and is now set up to form a salt bridge with Arg359 (Fig. 3D). Gly449 hydrogen-bonds to the carbonyl oxygen of Gly445 (Fig. 3E), and Ser450 to the carbonyl of Val446 (Fig 3F) and to the side chain of Thr452 (Fig. 3G). Finally, as mentioned, Thr453 binds to His 447 (Fig. 3H) to complete the ordering of the loop and to close in the active site for the second step of β -lactam formation.

Heartened by the 20-fold diastereomeric preference for the (2'*S*)-Me-CEA **10a** by the V446A mutant, we also investigated whether further space could be made on the "*S*" side of the active site to allow for a group larger than methyl. A V446G mutant was prepared, and the same synthetic pathway as above was followed to synthesize the 2'-ethyl CEA derivatives (**16**, Scheme 3), but first, compounds **17a** and **b** had to be prepared as shown in Scheme 4. Evans chiral auxiliary **18** was coupled with butyric acid to give compounds **19**. The benzyl group of the auxiliary was utilized to direct nucleophilic attack on benzyloxymethyl chloride (BOM-Cl), leading, after deprotection of **20**, to the desired enantiomers of **17**.

As shown in Table 1, across the board, the 2'-Et-CEA diastereomers **16** performed very poorly; their turnover rates were so low that K_M values could not be determined, and the values listed in Table 1 are only estimates of k_{cat} . Returning to the 2'-Me-CEA diastereomers **10**, V446G showed a stereochemical preference for these substrates similar to that of V446A, but its efficiency as measured by relative k_{cat}/K_M was lower by ~7-fold for **10a** and ~11-fold for **10b** (Table 1).

While the substitution of alanine for Val446 is likely to maintain the local secondary structure and simply create a "pocket" to accommodate the 2'-Me-CEA **10a**, replacement by the helix-breaker glycine is more problematic with respect to its effect on the local peptide conformation and entails, in any event, a smaller active site volume change than valine to alanine (methyl to hydrogen vs. isopropyl to methyl). With these considerations in mind, progression of the active site to accommodate a 2'-ethyl substituent could not be accomplished by simple first-sphere modification of the enzyme. It is possible that combinatorial alterations of the first- and second-sphere residues at this locus by, for example, cassette mutagenesis might achieve the desired outcome. This would be of interest from the perspective of rational engineering of enzyme active sites and the presumed need in this instance for only a slightly larger "pocket" to efficiently bind a (2'*S*)-ethyl substituent as a base for semi-synthetic clavulanates with Class C β -lactamase inhibitory properties.

Conclusions

Collectively, these results illustrate not only the virtue of X-ray structural information about an active site at various time points along the reaction coordinate but also the value of coupled kinetics experiments that link structural and dynamic information to guide protein

engineering. The substitution V446A in β LS created sufficient additional volume in the active site to achieve a 20:1 selectivity for one diastereomer of 2'-Me-CEA over the other with little or no loss of catalytic efficiency compared to the WT substrate with the mutant protein. The importance of opening and closing of catalytic loops, lids, flaps, and so forth in enzyme function is further underscored in this example both to allow chemistry to occur and to enable products to be released. Interference with even a single residue intimately involved in the structural integrity and behavior of a mobile loop, His447 in this class of enzymes, illustrates an important constraint on the rational choice of sites for mutational change. On the other hand, conservative mutations of residues not tied to structure or dynamics, such as V446A, can lead to useful selectivity among substrates, as we have shown here. In principle such planning can inform analysis of the relationship of protein structure and dynamics to catalysis and its rational manipulation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Drs. Kristos A. Moshos and Jonathan R. Scheerer for synthetic advice and Dr. Micah J. Bodner for early guidance with the kinetic assays. We are also deeply grateful to Dr. I. Philip Mortimer and Katherine Belecki for MS data, and to the NIH for financial support (AI014937).

References

1. Drawz SM, Bonomo RA. Clin Microbiol Rev. 2010; 23:160–201. [PubMed: 20065329]
2. Basker MJ, Bateson JH, Baxter AJ, Ponsford RJ, Roberts PM, Southgate R, Smale TC, Smith J. J Antibiot (Tokyo). 1981; 34:1224–1226. [PubMed: 7328063]
3. Golemi D, Maveyraud L, Ishiwata A, Tranier S, Miyashita K, Nagase T, Massova I, Mourey L, Samama JP, Mobashery S. J Antibiot (Tokyo). 2000; 53:1022–1027. [PubMed: 11132946]
4. Maveyraud L, Mourey L, Kotra LP, Pedelacq JD, Guillet V, Mobashery S, Samama JP. J Am Chem Soc. 1998; 120:9748–9752.
5. Beadle BM, Shoichet BK. Antimicrob Agents Chemother. 2002; 46:3978–3980. [PubMed: 12435704]
6. Reading C, Cole M. J Enzyme Inhib. 1986; 1:83–104. [PubMed: 3334242]
7. Bachmann BO, Li R, Townsend CA. Proc Natl Acad Sci U S A. 1998; 95:9082–9086. [PubMed: 9689037]
8. Bachmann BO, Townsend CA. Biochemistry. 2000; 39:11187–11193. [PubMed: 10985764]
9. McNaughton HJ, Thirkettle JE, Zhang ZH, Schofield CJ, Jensen SE, Barton B, Greaves P. Chem Commun. 1998:2325–2326.
10. Raber ML, Arnett SO, Townsend CA. Biochemistry. 2009; 48:4959–4971. [PubMed: 19371088]
11. Miller MT, Bachmann BO, Townsend CA, Rosenzweig AC. Proc Natl Acad Sci U S A. 2002; 99:14752–14757. [PubMed: 12409610]
12. Miller MT, Bachmann BO, Townsend CA, Rosenzweig AC. Nat Struct Biol. 2001; 8:684–689. [PubMed: 11473258]
13. Gerratana B, Stapon A, Townsend CA. Biochemistry. 2003; 42:7836–7847. [PubMed: 12820893]
14. Raber ML, Freeman MF, Townsend CA. J Biol Chem. 2009; 284:207–217. [PubMed: 18955494]
15. Raber ML, Castillo A, Greer A, Townsend CA. ChemBioChem. 2009; 10:2904–2912. [PubMed: 19882698]
16. Miller MT, Gerratana B, Stapon A, Townsend CA, Rosenzweig AC. J Biol Chem. 2003; 278:40996–41002. [PubMed: 12890666]
17. Bodner MJ, Phelan RM, Freeman MF, Li R, Townsend CA. J Am Chem Soc. 2010; 132:12–13. [PubMed: 20017478]

18. Sleeman MC, MacKinnon CH, Hewitson KS, Schofield CJ. *Bioorg Med Chem Lett*. 2002; 12:597–599. [PubMed: 11844680]
19. Salituro GM, Townsend CA. *J Am Chem Soc*. 1990; 112:760–770.
20. Miller AE, Bischoff JJ. *Synthesis-Stuttgart*. 1986:777–779.
21. Kinscherf TG, Willis DK. *J Antibiot (Tokyo)*. 2005; 58:817–821. [PubMed: 16506699]
22. Bodner MJ, Li R, Phelan RM, Freeman MF, Moshos KA, Lloyd EP, Townsend CA. *ChemBioChem*. 2011
23. Tahlan K, Park HU, Wong A, Beatty PH, Jensen SE. *Antimicrob Agents Chemother*. 2004; 48:930–939. [PubMed: 14982786]
24. Duchaud E, Rusniok C, Frangeul L, Buchrieser C, Givaudan A, Taourit S, Bocs S, Boursaux-Eude C, Chandler M, Charles JF, Dassa E, Derose R, Derzelle S, Freyssinet G, Gaudriault S, Medigue C, Lanois A, Powell K, Siguier P, Vincent R, Wingate V, Zouine M, Glaser P, Boemare N, Danchin A, Kunst F. *Nat Biotechnol*. 2003; 21:1307–1313. [PubMed: 14528314]

Fig. 1. “Snapshots” of the β -lactam synthetase reaction. Atoms are shown as sticks and colored by element. Enzyme carbons are colored purple; substrate, product, and intermediate carbons are shown in grey. Red spheres represent water molecules; green spheres magnesium ions. Faint grey lines indicate predicted hydrogen-bonds. All active sites shown are from subunit A of the crystal structures. (A) β LS complexed with ATP (PDB ID#1MB9). (B) β LS complexed with substrate CEA (5) and AMP-CPP (PDB ID#1JGT; CPP = methylenediphosphate). Note how Tyr348, Glu382, and Tyr326 have an alternate conformation from that in (A). (C) β LS complexed with PP_i and CMA-AMP (PDB ID#1MBZ; CMA = N^2 -carboxymethyl-L-arginine). The catalytic loop is shown as a purple tube. (D) β LS complexed with products DGPC (6), AMP, and PP_i (PDB ID#1MC1).

Fig. 2.

Hypothesized interaction of methyl-substituted tetrahedral intermediates. Modeled predictions of steric clashes in β LS involving the methyl substituent and either Val446 or His 447. Important residues are shown as sticks and colored by element. Enzyme carbons are colored purple; substrate and intermediate carbons are colored grey. Red spheres represent water molecules; green spheres represent magnesium ions. Green lines are predicted hydrogen-bonding interactions. A surface potential is shown as an orange mesh surrounding Val446 and His447. (A) (2' *R*)-Me-CEA (**10b**) clashes with His447. (B) (2' *S*)-Me-CEA (**10a**) clashes with Val446. (C) A V446A mutant frees (2' *S*)-Me-CEA from steric hindrance.

Fig. 3. Interactions predicted to affect and/or stabilize catalytic loop ordering. See text for a description of each step.

Scheme 1.
Biosynthesis of the 2-azetidinone moiety of clavulanic acid.

Scheme 2.

Comparison of clavulanic acid and carbapenem biosynthetic pathways.

Scheme 3.
 Synthesis of 2'-alkyl-CEA diastereomers.

Scheme 4.

Scheme 4 Synthesis of 2-hydroxymethylbutyric acids.

Table 1

Kinetic data of the β -lactam synthetase reaction.^a

Substrate	WT		V446A		V446G		H447A	
	K_M (mM)	k_{cat} (s ⁻¹)	K_M (mM)	k_{cat} (s ⁻¹)	K_M (mM)	k_{cat} (s ⁻¹)	K_M (mM)	k_{cat} (s ⁻¹)
CEA (5)	0.037 ± 0.001	0.87 ± 0.01	0.36 ± 0.02	0.28 ± 0.01	0.61 ± 0.06	0.13 ± 0.01	0.9 ± 0.03	0.016 ± 0.001
(2′ <i>S</i>)-Me-CEA (10a)	0.06 ± 0.01	0.023 ± 0.001	0.46 ± 0.04	0.34 ± 0.02	1.2 ± 0.1	0.13 ± 0.01	0.08 ± 0.06	0.013 ± 0.001
(2′ <i>R</i>)-Me-CEA (10b)	0.04 ± 0.01	0.032 ± 0.002	0.33 ± 0.02	0.014 ± 0.001	1.9 ± 0.7	0.07 ± 0.002	—	~0.003
(2′ <i>S</i>)-Et-CEA (16a)	0.06 ± 0.08	0.0023 ± 0.0006	~0.04	~0.004	—	~0.002	—	~0.001
(2′ <i>R</i>)-Et-CEA (16b)	—	~0.004	—	~0.004	—	—	—	—

^aThe uncertainties shown are standard deviations.
 —, too low to be measured or unable to calculate value within reasonable error