Pergamon

Bioorganic & Medicinal Chemistry Letters 12 (2002) 597–599

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

Enzymatic Synthesis of Monocyclic β-Lactams

Mark C. Sleeman, Colin H. MacKinnon, Kirsty S. Hewitson* and Christopher J. Schofield*

The Dyson Perrins Laboratory and the Oxford Centre for Molecular Sciences, South Parks Road, Oxford OX1 3QY, UK

Received 18 October 2001; accepted 26 November 2001

Abstract—An Mg^{2+} and ATP dependent β-lactam synthetase (BLS) catalyses formation of a β-lactam ring during the biosynthesis of clavulanic acid, an important β-lactamase inhibitor. An epimeric mixture of a 2-methylated derivative of the natural BLS substrate N^2-(2-carboxyethyl)-L-arginine was synthesised and found to be a substrate for the enzyme. The epimeric products were characterised by ^1H NMR and mass spectrometric analyses. The results suggest that a modified version of BLS might be used to catalyse the preparation of intermediates useful for the synthesis of β-lactam antibiotics.

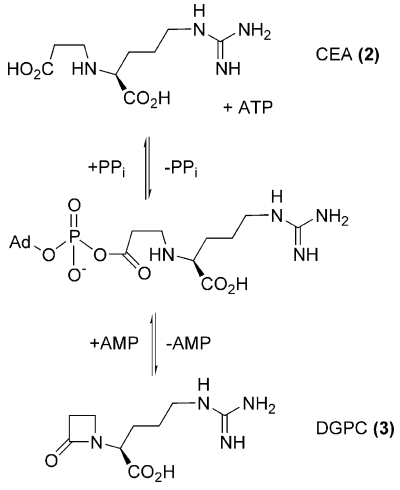
Clavulanic acid (**1**), an important inhibitor of serine β-lactamases, is produced by fermentation of *Streptomyces clavuligerus*. The biosynthetic pathway to clavulanic acid has been partly elucidated and a β-lactam synthetase (BLS) has been shown to catalyse the formation of the β-lactam ring.[1,2] In the BLS reaction N^2-(2-carboxyethyl)-L-arginine (CEA) (**2**) is cyclised to deoxyguanidinoproclavaminic acid (DGPC) (**3**), in a process driven by conversion of ATP to AMP and pyrophosphate (Scheme 1). A crystal structure for BLS has been recently reported.[3]

Clavulanic acid and many other β-lactam antibiotics/β-lactamase inhibitors based on the cephalosporins and penicillins are produced either directly by fermentation or from fermented materials already containing a β-lactam nucleus. Other sub-families of β-lactam antibiotics, such as the carbapenems, are produced via total synthesis.[4] The production costs of antibiotics and β-lactamase inhibitors, presently produced in the latter manner, limits their use and preclude development of new compounds.[5]

A key intermediate in the preparation of carbapenems and trinems is 3-(1-hydroxyethyl)-4-(acetyloxy)-2-azetidinone (**4**) which is produced by synthesis (Fig. 1).

In the absence of a fermentation method for the preparation of clinically used carbapenems and related antibiotics, it would be beneficial to have a fermentation route to **4** or an alternative common intermediate. Here, we report that recombinant wildtype BLS will accept an epimeric 2-methylated analogue, 2-methyl CEA (**6**), of its natural substrate (**2**), suggesting that modification of BLS to make synthetically useful β-lactams may be possible.

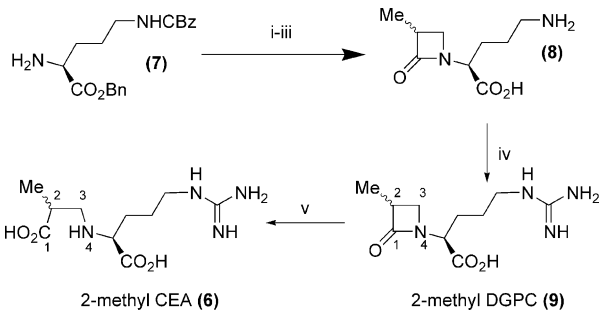
2-Methyl CEA (**6**) was prepared by substituting methyl acrylic acid for acrylic acid in the reported method for preparation of CEA (**2**).[6] Michael reaction of protected



Scheme 1. Formation of DGPC (**3**) from CEA (**2**).

*Corresponding author. Tel.: +44-1865-275625; fax: +44-1865-275674; e-mail: christopher.schofield@chem.ox.ac.uk; kirsty.hewitson@chem.ox.ac.uk

0960-894X/02/$ - see front matter
PII: S0960-894X(01)00806-X



Scheme 2. Synthesis of 2-methyl CEA (**6**) and 2-methyl DGPC (**9**). Reagents: (i) $CH_2{=}CMeCO_2H$, MeCN, 60 °C, 60%; (ii) $MeSO_2Cl$, $NaHCO_3$ (aq), MeCN, 60 °C; (iii) 10% Pd/C/H_2, EtOH/H_2O (2:1); (iv) 1-amidino-3,5-dimethylpyrazole–HNO_3, dimethylformamide–H_2O, pH 8–9, 45% plus 40% recovered **8**; (v) 1 M HCl, 1 h.

ornithine (**7**) with 2-methyl acrylic acid, followed by ring closure and deprotection, gave epimeric monocyclic β-lactam (**8**). Guanylation followed by hydrolysis gave the desired analogue (**6**) as a mixture of C-2 epimers (Scheme 2).

The *bls* gene has previously been expressed at relatively low levels using a pET24a(+) construct and purified by an involved two column protocol giving low overall yields.[2,7] To facilitate purification a polyhistidine tagged form of BLS was produced by cloning *bls* into the pET28a(+) vector (from Novagen). BLS produced using this vector was isolated to >95% purity (by SDS–PAGE analysis) using a single affinity purification step with a Nickel His·Bind™ column (Fig. 2).[8]

The 2-methylated substrate analogue (**6**) was initially assayed as a substrate for BLS using a fluorescence detection pre-column derivatised HPLC assay based on the work of Kai et al.[9,10] Under the standard assay conditions ⩾50% of the 2-methyl CEA (**6**) was converted to 2-methyl DGPC (**9**), which was observed as a single HPLC peak coincident with that produced when

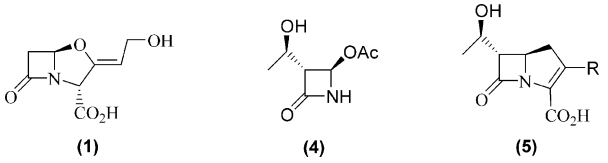


Figure 1. Structures of clavulanic acid (**1**), 3-(1-hydroxyethyl)-4-(acetyloxy)-2-azetidinone (**4**) and a carbapenem (**5**).

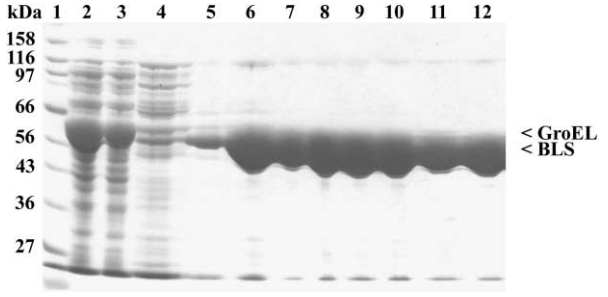


Figure 2. SDS–PAGE gel analysis of purified BLS. (1) Molecular weight markers (NEB). (2) Crude cell lysate, soluble fraction. (3) Flow through on loading His·Bind column. (4) Flow through on washing His·Bind column. (5)–(12) Fractions from protein elution.[8]

using authentic **9**. The HPLC assays were also carried out using wildtype BLS to ensure the polyhistidine tag was not affecting the conformation of the protein such that it accepted the alternative substrate. A similar result was obtained. Incomplete substrate conversion may in part result from partial epimerisation at the α-centre derived from arginine during the ring closure process in the synthesis.[11] A larger scale incubation was used to allow analysis by ^{1}H NMR (500 MHz). Peaks were observed at δ 4.2–4.5 consistent with conversion of ATP to AMP (Fig. 3).

The appearance of peaks corresponding to 2-methyl DGPC product (**9**) was also observed, including δ 4.05 (epimer 1, dd, *J* 10.0, 4.5Hz, C**H**CO_2H), δ 4.0 (epimer 2, dd, *J* 9.5, 5.5 Hz, C**H**CO_2H) and δ 1.5–2.0 ($CH_2CH_2CH_2N$ and $CH_2CH_2CH_2N$) (Fig. 4).

The β-lactam product was isolated by reverse phase HPLC (C18 250 × 10 mm). Samples were eluted isocratically with a mobile phase of 10% (v/v) MeOH at a flow rate of 4 mL/min. ESI MS analysis of the product was consistent with the production of 2-methyl DGPC (**9**) (*m*/*z* 243 $[M-H]^+$). ^{1}H NMR (500 MHz), including 2D ^{1}H COSY analysis of the product, was consistent with the formation of both 2-methyl epimers of the product. There appeared to be a slight excess (<5%) of one epimer in the starting material (**6**) (by ^{1}H NMR analysis); a similar excess was apparently also present in the enzymic product (**9**), suggesting that both epimers are approximately equally efficient substrates for BLS.

BLS is only the second enzyme after isopenicillin-*N*-synthase to have been shown to be able to catalyse formation of a β-lactam ring using an unnatural substrate.[12]

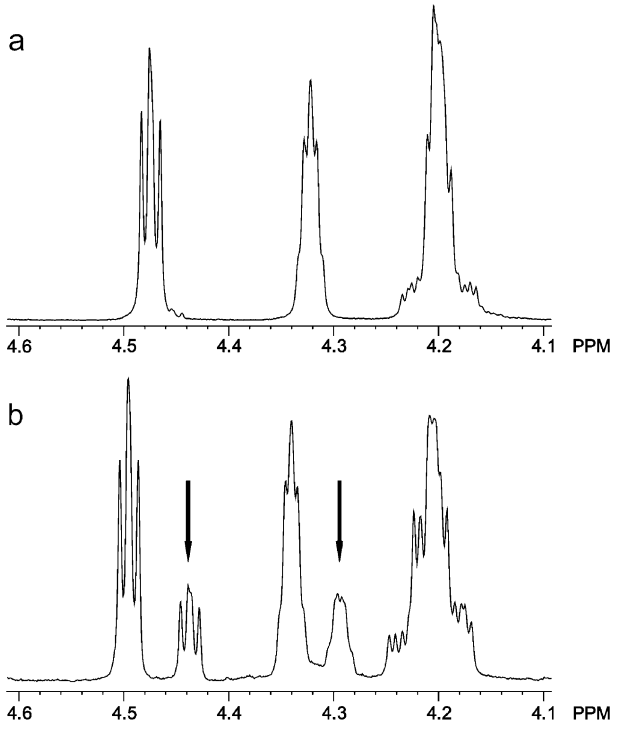


Figure 3. Partial ^{1}H NMR spectra (500 MHz, D_2O) of (a) ATP and (b) assay mixture showing conversion to AMP (arrowed).

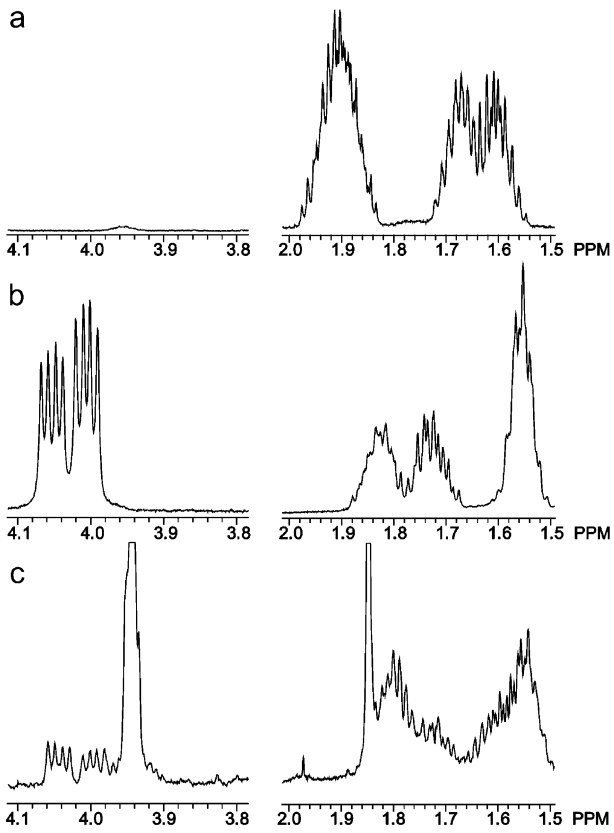


Figure 4. Selected regions of the ^{1}H NMR spectra (500 MHz, D_2O) of (a) 2-methyl CEA (**6**), (b) 2-methyl DGPC (**9**) and (c) assay mixture.

Somewhat surprisingly, both the epimers of 2-methyl CEA (**6**) appear to be (approximately) equally good substrates for BLS. This result is encouraging for attempts to modify BLS by mutation to mediate the formation of β-lactams with a hydroxyethyl functionality at C-2 and a readily functionalisable (e.g., CH_2CO_2H) or cleavable group on the lactam nitrogen.

Acknowledgements

We thank the BBSRC, EPSRC, MRC and the EU for funding, and Drs. B. Odell and R. T. Aplin for NMR and mass spectrometric analyses.

References and Notes

1. Bachmann, B. O.; Li, R. F.; Townsend, C. A. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 9082.
2. McNaughton, H. J.; Thirkettle, J. E.; Zhang, Z. H.; Schofield, C. J.; Jensen, S. E.; Barton, B.; Greaves, P. *J. Chem. Soc., Chem. Commun.* **1998**, 2325.
3. Miller, M. T.; Bachmann, B. O.; Townsend, C. A.; Rosenzweig, A. C. *Nat. Struct. Biol.* **2001**, *8*, 684.
4. Page, M. I. *The Chemistry of beta-Lactams*, 1st ed.; Blackie Academic & Professional: London, 1992.
5. Schofield, C. J., Walter, M. W. In *Amino Acids, Peptides and Proteins*; Davies, J. S., Ed.; Royal Society of Chemistry: Cambridge, UK, 1999; Vol. 30, p 335.
6. Baldwin, J. E.; Lloyd, M. D.; Whason, B.; Schofield, C. J.; Elson, S. W.; Baggaley, K. H.; Nicholson, N. H. *J. Chem. Soc., Chem. Commun.* **1993**, 500.
7. Bachmann, B. O.; Townsend, C. A. *Biochemistry* **2000**, *39*, 11187.
8. The *bls* gene in the pET28a(+) vector and a plasmid bearing the chaperonin GroELS[13] were simultaneously transformed into *Escherichia coli* BL21(DE3) for co-expression. Cells were grown in 2TY containing kanamycin and 30 μg/mL chloramphenicol at 37 °C. When the absorbance at 600 nm reached ~1.0, IPTG was added to a final concentration of 1.0 mM and the cells harvested 16 h later by centrifugation. Expression of BLS was observed as 15% of the total cell protein in the soluble fraction (by SDS–PAGE analysis). Cells were resuspended in binding buffer (20 mM Tris–HCl, pH 7.9, 500 mM NaCl, 5 mM imidazole) and lysed by sonication. The lysate was centrifuged at 40,000 *g*, and the supernatant loaded onto a pre-equilibrated 10 mL His·Bind™ column. The column was washed with 100 mL binding buffer followed by 60 mL wash buffer (binding buffer with 60 mM imidazole). Protein was eluted with elute buffer (binding buffer with 200 mM imidazole). The protein was desalted through a PD-10 column (Pharmacia) and concentrated to ~10 mg/mL. BLS was aliquoted into 50 μL portions and stored at −80 °C.
9. Kai, M.; Miyazaki, T.; Yamaguchi, M.; Ohkura, Y. *J. Chromatogr.* **1983**, 425.
10. Assay mixtures contained: 150 mM Tris–HCl, pH 9, 5 mM ATP, 10 mM $MgCl_2$, 2 mM 2-methyl CEA, ca. 74 μg enzyme. Reactions were incubated at 37 °C for 15 min and then derivatised. Derivatisation conditions: To 50 μL assay mixture were added 25 μL benzoin (40 mM) in 2-methoxyethanol, 25 μL sodium sulphite (0.2 M)/β-mercaptoethanol (0.1 M), 50 μL KOH (2 M). The mixture was cooled on ice for 2 min, heated to 100 °C for 5 min and then cooled on ice for a further 2 min. 50 μL of a 1:1 mixture of HCl (4 M), and Tris HCl (1 M, pH 9.2) was then added and the sample centrifuged for 30 seconds at 11,000 *g*. 150 μL of the above sample was injected on to a reverse phase Hypersil phenyl column (250 × 4.6 mm) and the elution of compounds compared to that of standards. A linear gradient of 15% (v/v) Tris–HCl (0.5 M), pH 8.5, 50–80% (v/v) MeOH over 16 min remaining at 80% (v/v) MeOH for 6 min, reverting to 50% (v/v) MeOH over 1 min and re-equilibrating under these conditions for a further 15 min, was run at 0.7 ml/min. Fluorescence was measured at 425 nm against 325 nm excitation.
11. Baldwin, J. E.; Merritt, K. D.; Schofield, C. J.; Elson, S. W.; Baggaley, K. H. *J. Chem. Soc., Chem. Commun.* **1993**, 1301.
12. Baldwin, J. E.; Bradley, M. *Chem. Rev.* **1990**, *90*, 1079.
13. Azem, A.; Kessel, M.; Goloubinoff, P. *Science* **1994**, *265*, 653.