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Enantioselective Syntheses of 1-Carbacephalosporins from Chemoenzymically Derived β-Hydroxy-α-Amino Acids: Applications to the Total Synthesis of Carbacephem Antibiotic Loracarbef

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The authors wish to dedicate this article to the memory of Dr Lowell D. Hatfield, who lost his life in the fight against lung cancer on 13 June 1999

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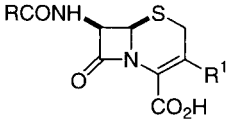
Abstract—Serine hydroxymethyltransferase (SHMT) derived from recombinant *E. coli* was found to be able to catalyze the condensation between glycine and 4-pentenaldehyde, affording enantiopure L-*erythro*-2-amino-3-hydroxy-6-heptenoic acid (AHHA) in high yield and throughput. Conversion of this chiral intermediate of biosynthetic origin to the oral carbacephalosporin antibiotic loracarbef (Lorabid®) via β-lactam forming reactions and subsequent Dieckmann cyclization was achieved. © 2000 Elsevier Science Ltd. All rights reserved.

Introduction

Cephalosporins (**1**) have been the most widely used antibiotics in the world to eradicate bacterial infections.[1–8] Replacement of the sulfur atom by a methylene in cephalosporins has led to a new class of antibiotics, i.e. carbacephalosporins (**2**), which were shown to exhibit remarkable enhancement of chemical and serum stability over their parent compounds.[9–11] These include several antibiotics and β-lactamase inhibitors. However, unlike their sulfur counterparts which are usually obtained by partial synthesis from either penicillin sulfoxide esters or side chain modification of natural cephalosporins, carbacephalosporins are almost exclusively obtained by de novo total synthesis.



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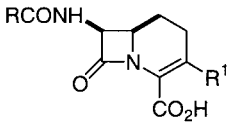
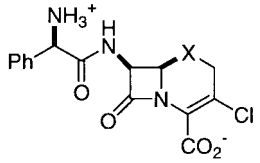


2

R^1 = H, Cl, alkenyl or heteroaryls

R = *D*–α-aminoarylmethyl, or heteroaryl oxime groups



3 cefaclor (Celclor,® X = S)

4 loracarbef (Lorabid,® X = CH_2)

The inherent chiral centers at the ring juncture, strained [4.2.0] bicyclic system and a high density of functional groups make these molecules very challenging targets for large scale manufacture. The difficulty in the synthesis contributed, in no small part, to the fact that despite their advantageous biological activities demonstrated both in vivo and in vitro, the only carbacephalosporin that has

Keywords: aldolase; antibacterials; biocatalysis; cephalosporins; carbacephalosporins; β-hydroxy-α-amino acid; β-lactam; loracarbef; serine hydroxymethyltransferase; SHMT.

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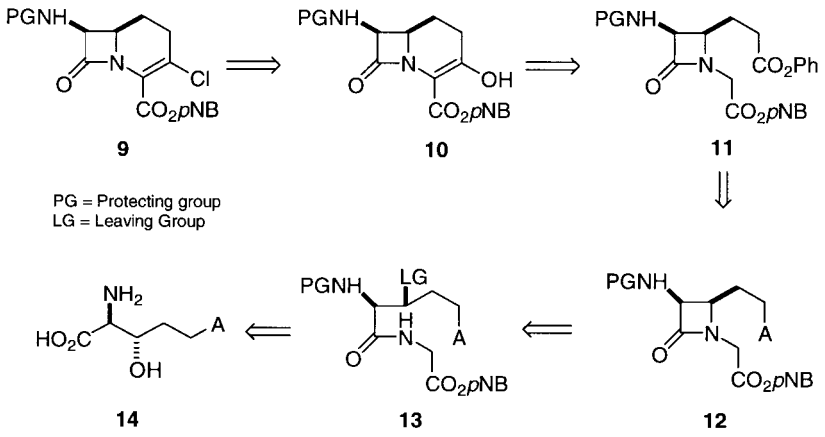
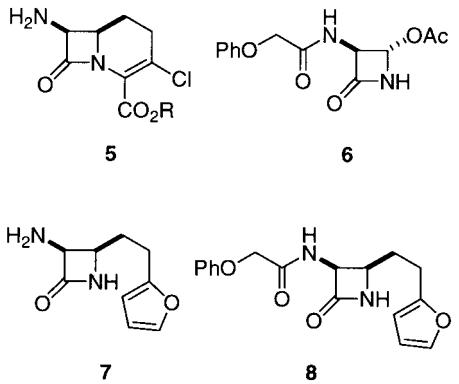


Scheme 1.

been developed and approved for clinical usage is loracarbef (**2**, R=*D*-phenylglycinoyl, R^1=Cl), discovered by scientists at Kyowa Hakko,[12] developed and marketed by Eli Lilly as an orally active and broad spectrum antibiotic.[13]

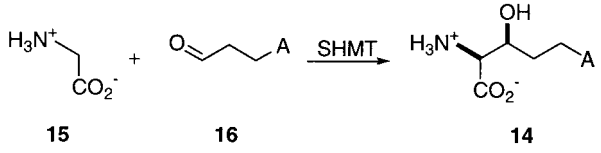
The first laboratory-scale synthesis was achieved by an intramolecular Horner–Emmons–Wadsworth condensation to give the racemic nucleus **5** (R=*t*-butyl), which was then kinetically resolved via enzymatic acylation with phenylglycine.[14] An exploratory scale synthesis from these laboratories involved a partial synthesis from **6**, whose genesis from penicillin sulfoxide was known.[15] One of the first kilogram-scale syntheses of loracarbef utilized the oxazolidinone chiral auxiliary of Evans and Sjogren[16–18] for synthesis of **8**. Subsequent elaboration of **8** to nucleus **5** (R=*p*-nitrobenzyl) involved appending the six-membered ring via rhodium-mediated carbenoid insertion. An improved synthesis of **4** was developed later and utilized a highly efficient enzymatic kinetic resolution of 3-amino-azetidinone **7**.[19,20] A further improvement in converting **8** to **5** (R=*p*-nitrobenzyl) featured a regiospecific Dieckmann cyclization for annulation of the tetrahydropyridine.[22,50] Other published syntheses of loracarbef have utilized penicillin sulfoxide ester derived intermediates[23,24] and an enantioselective synthesis starting from sodium erythorbate.[25,26] Still, a more streamlined and economical synthetic process that produces high quality loracarbef in a robust, safe, and environmentally friendly manner is highly desirable.



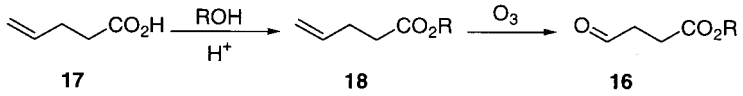
Result and Discussions

We envisioned that a protected loracarbef nucleus **9** (PG=amino protecting group) could be prepared from a β-hydroxy-α-amino acid derivative **14** (A=latent carboxylate synthon) as outlined in Scheme 1 in a retrosynthetic sense. The choice of *p*-nitrobenzyl ester was based on its ready removal and the need to control the quality of the final product by not introducing new impurities. Use of β-hydroxy-α-amino acid derivatives for synthesis of monocyclic β-lactams was pioneered by Miller[21,27–30] and was used thereafter by Squibb scientists for the synthesis of monocyclic antibiotics.[12] Applications of variously substituted β-hydroxy-α-amino acid derivatives for synthesis of bicyclic β-lactams was again reported by Miller[33,34,51] and in preliminary publications from these laboratories. However, to achieve the proposed synthesis of loracarbef in an enantioselective manner, four major challenges emerged: First, an enantiopure or reasonably enriched source of compound **14** had to be secured. Secondly, an efficient methodology for the construction of the β-lactam ring needed to be achieved to afford the monocyclic compound **12**. Thirdly, a suitable carboxylate synthon had to be incorporated at an appropriate stage of the synthesis to allow for the Dieckmann cyclization. Last, but not least, a proper amino protecting group (PG) would have to be chosen to enable high chemoselectivity throughout the process and to ensure facile removal at the end to reveal the highly functionalized molecule.

While a number of methods are available for the synthesis of compounds represented by the general formula **14**, most of them suffer from disadvantages including long and tedious synthesis, use of expensive chiral auxiliaries and involve chromatographic purification processes. As a starting material for the synthesis of a drug substance in



Scheme 2.



Scheme 3.

multi-ton quantities, ready availability is a prerequisite. We anticipated being able to use the enzyme serine hydroxymethyltransferase (SHMT)[54] for construction of the desired stereoisomer of **14**, as shown in Scheme 2. Serine hydroxymethyltransferase (SHMT) (E.C.2.1.2.1) is a widely occurring enzyme that catalyzes the physiological conversion of serine (Ser) to glycine (Gly), transferring the hydroxymethylene unit to tetrahydrofolate;[35–42] however, it has been employed in the reverse direction for synthesis of chiral β-hydroxy-α-amino acids[42,43] from glycine and various aldehydes. To test the viability of the chemistry shown in Scheme 2, we required developmental quantities of the enzyme which was known to be available from a variety of natural sources. Initial quantities of the enzyme were isolated from rabbit liver using the methodology of Martini et al.[44–46] In later studies the enzyme was derived via expression of the cloned *gly A* gene from *Escherichia coli*.[47] As the desired SHMT enzyme accounts for a large portion of the crude protein mass isolated from the *E. coli*, the fermentation broth of the crude enzyme can be used as such without the need of further purification.

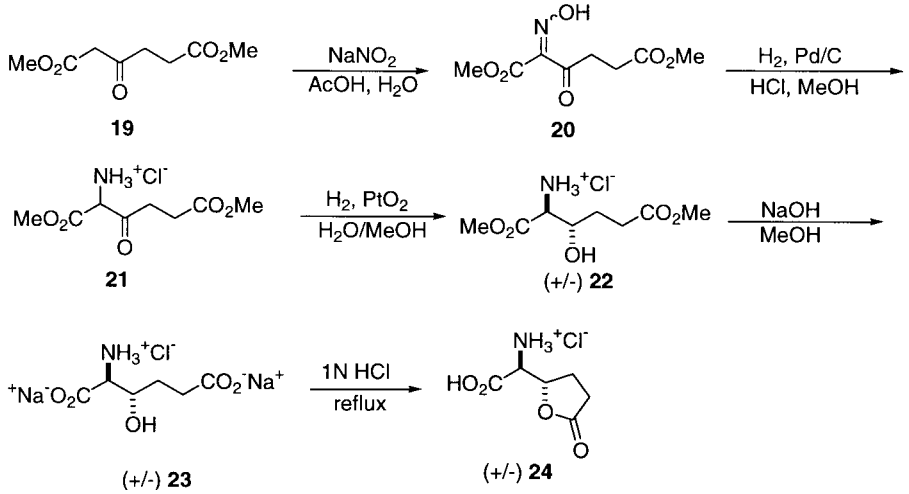
We initially chose the esters of succinic semialdehyde **16** (A=CO_2Me, CO_2Et, CO_2^tBu) as potential substrates for the enzymic reaction. Synthesis of the various substrates was achieved by esterification of 4-pentenoic acid followed by ozonolysis (Scheme 3). Miller[42] had shown that the methyl ester of succinic semialdehyde was a good substrate for the enzymic synthesis. Additionally, evidence for the product being the desired L-*erythro* stereoisomer was circumstantial.

For analysis of reaction mixtures arising from the enzyme-mediated reaction of glycine with various aldehydes, an HPLC methodology was developed based on a procedure reported by Aswad[48] for the separation and quantification of D- and L-aspartic acid. The procedure involved derivatization of the amino acid with *o*-phthaldehyde and *N*-acetyl-L-cysteine. This methodology, by providing a chromophore (isoindole) and a second chiral carbon atom, was able to convert a racemic mixture of amino acids into a mixture of diastereomers which were separable via conventional reverse phase HPLC and could be detected using a normal UV detector. However, for unequivocal stereochemistry assignment of the products from the initial enzyme screening, all four possible stereoisomers of **14** (A=CO_2R) were needed.

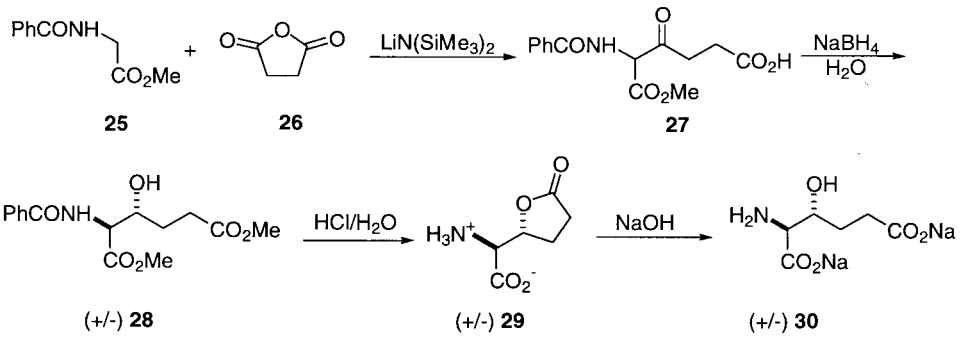
Synthesis of the racemic *erythro* stereoisomer is shown in Scheme 4. The selective hydrogenation of oxime **20**[49] afforded ketoamino acid **21**, which was further reduced by diastereoselective hydrogenation under platinum oxide to give *erythro* β-hydroxy-α-amino acid **22** in racemic form. The free diacid derived from **22** was found to be prone to cyclization under acidic conditions to give lactone **24**. However, both compound **23** and **24** proved useful as analytical standards. Availability of these compounds not only facilitated analytical aspects of the biocatalysis reaction but also provided valuable intermediates, allowing the evaluation of downstream chemistry concurrent with the development of the enzymatic process.

The corresponding racemic *threo* stereoisomers were synthesized as shown in Scheme 5. Methyl hippurate was condensed with succinic anhydride giving **27**, which was reduced with sodium borohydride to give *threo* **28**. Attempts to hydrolyze both the *N*-benzoyl blocking group and the methyl ester of **28** by saponification were not successful; however, hydrolysis in aqueous hydrochloric acid did effect the desired hydrolyses. The product of the acid-mediated hydrolysis was lactone **29**, which could be hydrolyzed to give the racemic *threo* stereoisomer **30**.

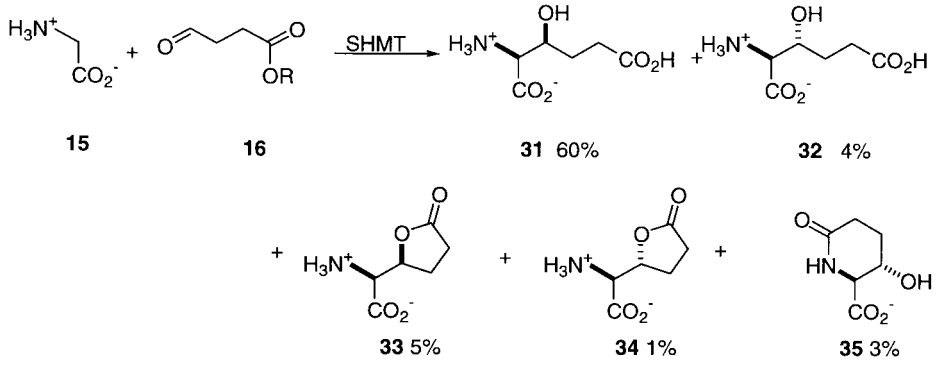
With these authentic isomers of α-amino-β-hydroxyadipic



Scheme 4.



Scheme 5.



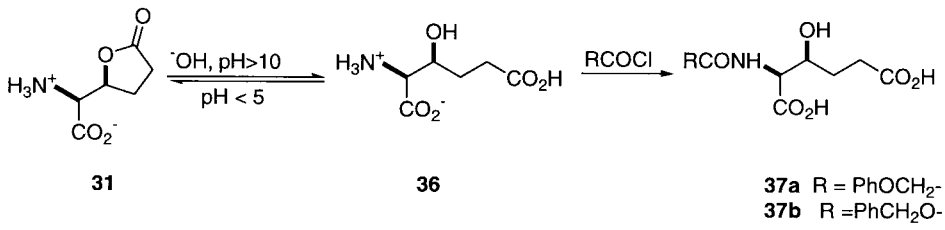
Scheme 6.

acid derivatives in hand and analytical methodology in place, compound **16** was subjected to condensation with excess glycine in the presence of the enzyme SHMT. Indeed, L-*erythro* acid **31** (2*S*, 3*S*) was obtained in good yield, along with a small amount of L-*threo* (2*S*, 3*R*) isomer **32**, and their corresponding lactones (**33**, **34**) and lactam (**35**, Scheme 6).

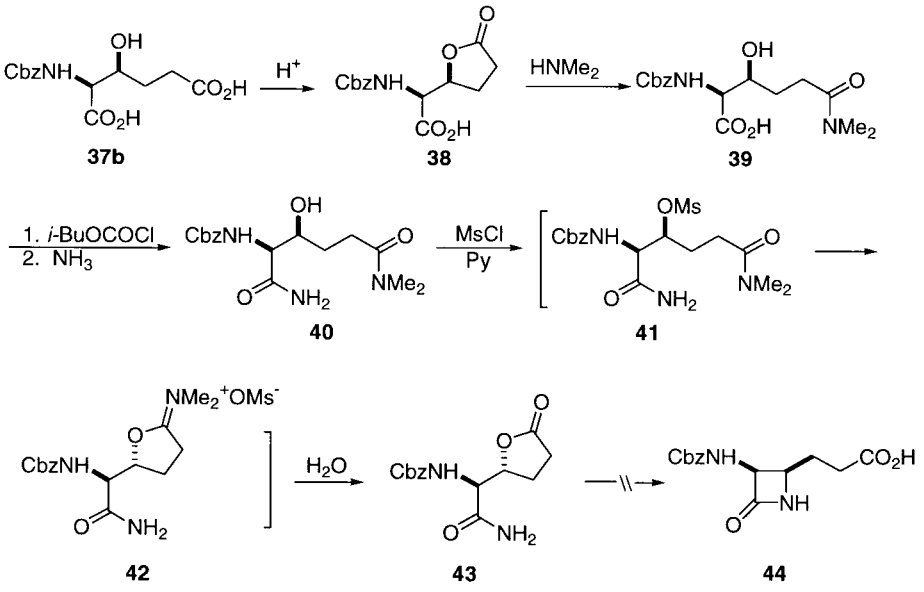
The lactones, however, can be converted into open chained diacids. Isolation of these highly water soluble compounds from the aqueous enzymatic reaction media was facilitated by converting the free amino acids in situ into the corresponding amide **37a** or carbamate **37b**, respectively (Scheme 7).

Further transformation of these compounds into the desired β-lactam **44** proved very problematic. While the distal carboxylic group can be differentiated and selectively converted into dimethyl amide **39** by way of lactone **38**, numerous attempts to effect β-lactam ring closure through nucleophilic displacement of the hydroxyl group by the primary amide nitrogen failed. In most cases the distal amide oxygen proved to be more nucleophilic than the intended primary amide nitrogen and as a consequence, amidate salt **42** was isolated as a stable solid, which upon hydrolysis yielded lactone **43**. Efforts aimed at converting compound **43** into lactam **44** met with little success as a poor nucleophile (amide nitrogen) failed to displace an equally poor leaving group (lactone oxygen, Scheme 8). Apparently two issues had to be resolved: First, the distal carboxylate needed to be prevented from participating in the cyclization event. Secondly the nucleophilicity of the amide nitrogen needed to be enhanced.

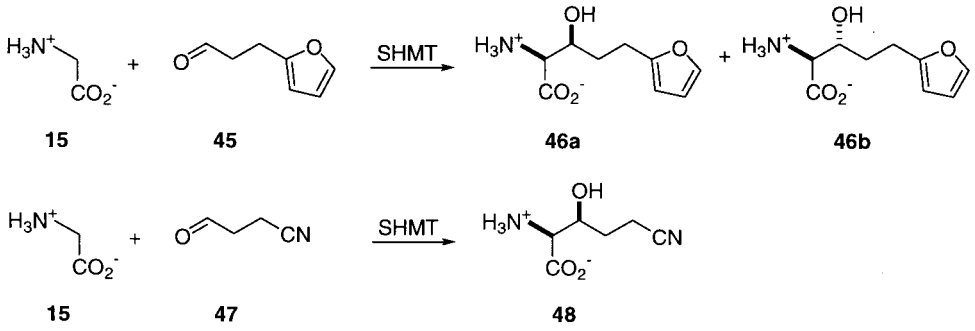
To address the first concern, compound **45**, an aldehyde with a furan ring attached as a more benign carboxylate synthon, and glycine were subjected to the enzyme catalyzed aldol condensation. The anticipated product, β-hydroxy-α-amino acid, also had the added advantage of being convertible into pivotal intermediates (**7** and **8**) of a previous large scale



Scheme 7.

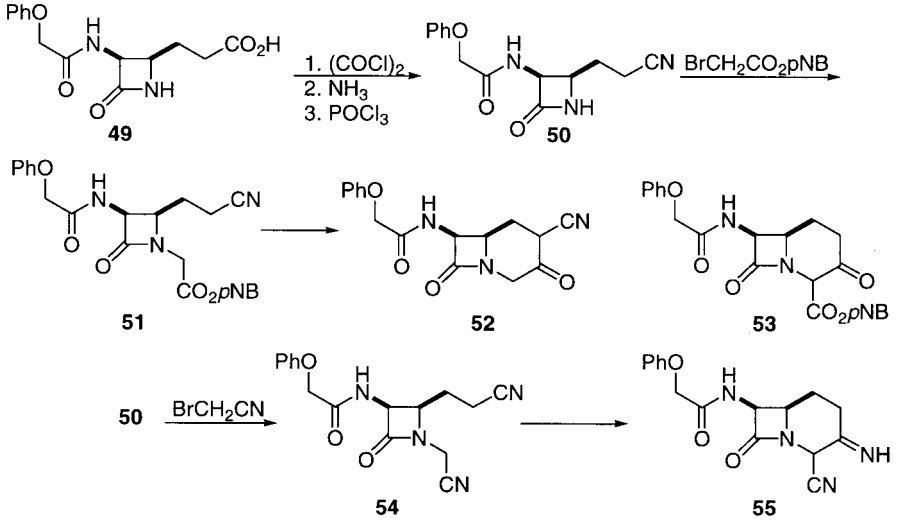


Scheme 8.

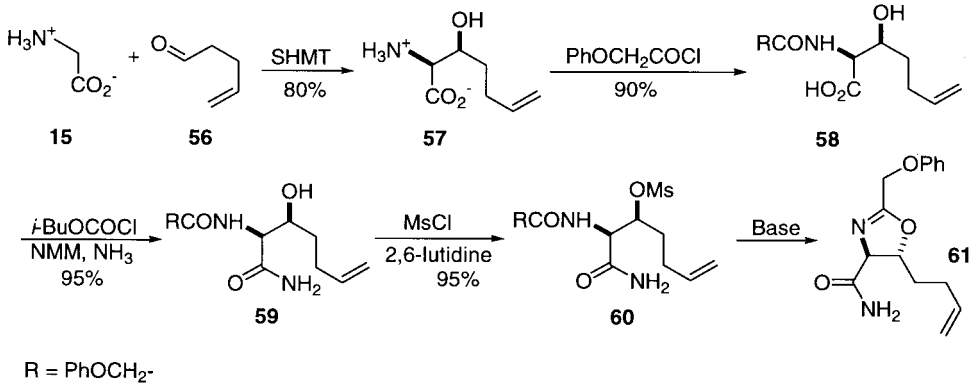


Scheme 9.

synthesis. To our disappointment, **45** turned out to be a poor substrate for SHMT, causing severe enzyme inhibition. Moreover, diastereoselectivity of the reaction was very low and the resulting epimeric mixture **46** proved difficult to separate. Aldehyde **47**, on the other, showed much more promise. Besides being a readily available and inexpensive starting material, its reaction with glycine catalyzed by SHMT afforded cyanoamino acid **48** in high enantioselectivity and diastereoselectivity (Scheme 9). Since scale-up of the enzymatic reaction and carrying out the



Scheme 10.

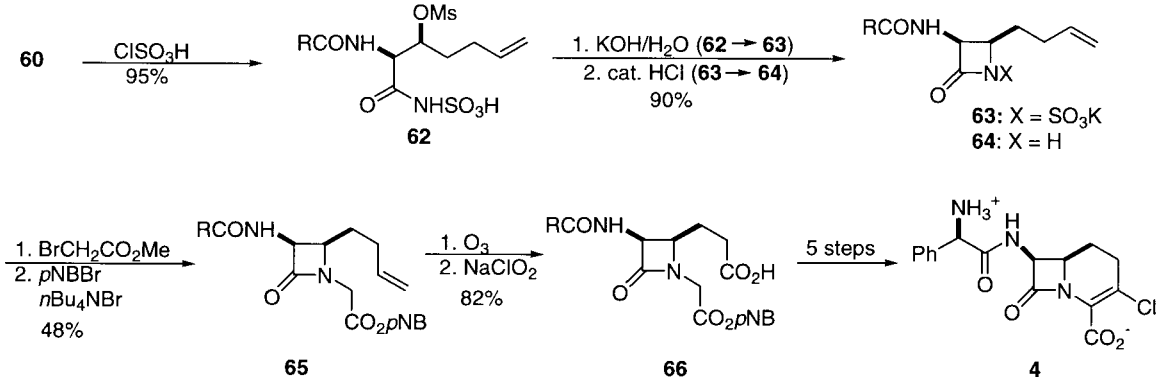


Scheme 11.

entire sequence was resource intensive, we prepared compound **50** from known acid **49** as a model to check the viability of using nitrile as a carboxylate equivalent in the total synthesis. It was quickly established that although the methylene protons adjacent to the carboxylate in **51** are more acidic than those next to the cyano group, the *p*-nitrobenzyl ester appeared to be a much better electrophile, and consequently, the only product isolated from the condensation reaction was keto nitrile **52**. This notion is further supported by the fact that when dinitrile **54** was subjected to the same reaction condition, iminonitrile **55** was formed in good yield. While this compound has the same carbon framework and functional group alignment as the much desired enol ester **10**, its conversion to the latter compound proved to be a formidable challenge due to the lability of the β-lactam ring under hydrolytic conditions (Scheme 10).

These early learning experiences underscored the need and importance of strategically addressing all four elements of the synthesis: (1) availability of the chiral synthon; (2) an efficient β-lactam forming step, a selective ring annulation; (3) a judicious choice of protecting groups; and (4) an efficient annulation protocol. This also led us to examine the use of an ethylene unit as a latent carboxyl group and to exploit the higher nucleophility of the amide nitrogen in its anionic form. To our delight, 4-pentenal (**56**) proved to be an excellent substrate for SHMT, and intense optimization efforts on both the enzyme expression and biocatalysis fronts yielded a set of condition that afforded amino acid **57** in high yield and selectivity. In situ protection of the amino acid was achieved in high yield to give **58** which was converted into mesylate **60**. Here again the nucleophilicity of the primary amide nitrogen was sufficient enough and most of the starting compound went on to give oxazoline **61**,[51] the same compound produced when alcohol **59** was subjected to Mitsunobu conditions.[52] However, displacement of the mesylate by the amide nitrogen was indeed possible by converting **60** into *N*-sulfonamate **62** using the Squibb protocol,[31,32] affording β-lactam **63** in high yield. Direct attachment of a *p*-nitrobenzyl acetate as preferred for the downstream chemistry was complicated by side products formation, primarily hydrolysis of the esters. However, the desired *p*NB ester **65** could be conveniently obtained by an in situ ester exchange reaction under phase transfer catalysis (Scheme 11).[53]

Several methods were explored for the conversion of the terminal alkene into a carboxylic acid, with an in situ oxidation of the aldehyde obtained by ozonolysis being the most preferable.[55–57] This protocol afforded carboxylic acid **66** in excellent yield. This compound was successfully converted into loracarbef in five synthetic steps as previously reported.[50] This consisted of sequential phenyl ester formation (PhOH, DMAP), Dieckmann cyclization (*t*BuOLi), chlorination–deacylation ($(PhO)_3P$, Cl_2), side-chain attachment and reductive *p*-nitrobenzyl ester removal, thus completing the total synthesis of this carbacephalosporin antibiotic from an unnatural chiral amino acid of biosynthetic origin (Scheme 12).



Scheme 12.

In summary, we have developed a new synthesis of loracarbef based on the feasibility of using serine hydroxylmethyl transferase (SHMT) as a catalyst for the enantioselective condensation between glycine and 4-pentenal. The synthesis entails a novel enantioselective biocatalysis step, and a chemoselective β-lactam forming reaction. As our goal was to develop an industrial process for the synthesis of this valuable antibiotic in large scale, much effort has been focused on improving the practical aspects of the chemistry and biology. It is evident that in order to realize the envisioned chemistry on large scale utilizing an enzyme which has rarely been used in synthesis, a collaborative effort between molecular biologists, biochemists, analytical chemists, organic chemists and chemical engineers must be coordinated to address various concerns in a coherent fashion. We have demonstrated the efficiency and potential of using isolated enzymes for enantioselectively establishing chiral centers via carbon–carbon formation, a process that would have involved a tedious protection–deprotection protocol, chiral auxiliary attachment–detachment or resolutions if more traditional approaches were employed. Applications of a SHMT catalyzed condensation reactions to the synthesis of other natural and synthetic molecules of biological importance as well as improved syntheses of loracarbef of similar genesis will be reported in due time.

Experimental

All reactions were carried out under a nitrogen atmosphere. Reactions were monitored by HPLC, TLC or GC. ^{1}H and ^{13}C NMR spectra were recorded on Bruker AC300 or GE QE300 instruments. IR-spectra were recorded on a Perkin–Elmer 281. Elemental analyses were performed by Eli Lilly Physical Chemistry Laboratories.

18. 4-Pentenoic acid (**17**, 25.0 g, 250 mmol) was cooled to 0°C and treated with oxalyl chloride (24.0 mL, 275 mmol) over a period of 0.75 h. The resulting mixture was stirred at 0–5°C for 1 h and then warmed to 35–40°C, during which time gas evolution was evident. After evolution of gas had ceased, the mixture was cooled to 0°C and treated with MeOH (40.6 mL, 999 mmol). This mixture was stirred at 0–5°C for 1 h and then warmed to room temperature. After 15 h, a distillation apparatus was put in place and the excess methanol was removed via distillation while keeping the pot temperature below 70°C. The resulting residue was dissolved in Et_2O (150 mL), washed with 5% $NaHCO_3$ (50 mL), brine (50 mL), and dried over $MgSO_4$. The solvent was removed in vacuo with no external heating of the bath to afford 20.9 g (73.3%) of **18** as a clear liquid. ^{1}H NMR ($CDCl_3$, 300 MHz): δ 2.33–2.45 (m, 4H), 3.67 (s, 3H), 4.98–5.09 (m, 1H), 5.76–5.89 (m, 2H).

16. Compound **18** (20.8 g, 182 mmol) was dissolved in CH_2Cl_2 (65 mL) and treated with Sudan III (5 mg). The solution was ozonized at −78°C until the disappearance of red color. Dimethyl sulfide (40.1 mL, 547 mmol) was added and the mixture allowed to warm to room temperature while stirring overnight. The solvent was removed in vacuo and the crude material dissolved in Et_2O (150 mL). This solution was filtered through a pad of florisil (50 g), which was then rinsed with Et_2O (50 mL). The filtrate was washed with brine (50 mL), dried over $MgSO_4$ and concentrated in vacuo to afford the crude **16** (19.9 g, 94%). The crude material was purified by distillation (61–63°C at 4 mmHg) to afford **16** as a clear oil (13.2 g, 62.5%). ^{1}H NMR ($CDCl_3$, 300 MHz): δ 2.62–2.66 (t, 2H), 2.79–2.83 (t, 2H), 3.70 (s, 3H), 9.82 (s, 1H).

20. Dimethyl 3-oxo-adipate (**19**) (5.0 g, 26.6 mmol) was dissolved in AcOH (10.0 mL), treated with H_2O (5 mL) and cooled to 0°C. The material was diazotized with $NaNO_2$ (2.8 g, 39.8 mmol) in H_2O (12 mL) by stirring at 0–5°C for 4 h. The reaction mixture was diluted with CH_2Cl_2 (50 mL), extracted with 5% $NaHCO_3$ (3×25 mL), and dried over $MgSO_4$. Concentration in vacuo afforded **20** as a yellow oil (5.1 g, 88.4%). ^{1}H NMR ($CDCl_3$, 300 MHz): δ 2.67–2.71 (t, 2H), 3.15–3.19 (t, 2H), 3.71 (s, 3H), 3.90 (s, 3H). ^{13}C NMR ($CDCl_3$, 75 MHz): δ 27.3, 32.4, 52.2, 52.3, 150.0, 161.9, 174.1, 193.7.

21. Compound **20** (1.0 g, 46.5 mmol) was dissolved in a mixture of MeOH (21 mL) and 1N HCl (4.9 mL, 49.0 mmol) and added to a Parr bottle containing 10% palladium on carbon (200 mg) under a nitrogen atmosphere. The resulting mixture was hydrogenated at 50 psi for 21 h using a Parr shaker. The reaction mixture was filtered through a pad of celite and concentrated in vacuo to afford **21** as a white solid (1.1 g, 100%). ^{1}H NMR (D_2O, 300 MHz): δ 1.86–2.07 (m, 2H), 2.25–2.40 (m, 1H), 2.50–2.65 (m, 1H), 3.16 (s, 1H), 3.18 (s, 3H). ^{13}C NMR (D_2O, 75 MHz): 30.3, 37.6, 55.1, 57.1, 166.9, 177.9, 179.4, 201.2.

22. Compound **21** (0.46 g, 19.2 mmol) was dissolved in a mixture of MeOH (15 mL) and H_2O (10 mL) and added to a Parr bottle containing platinum oxide hydrate (30 mg). The resulting mixture was hydrogenated at 50 psi for 15 h using a Parr apparatus. The reaction mixture was filtered through a pad of celite and concentrated in vacuo to afford **22** as a thick oil (0.46 g, 98%). ^{1}H NMR (D_2O, 300 MHz): δ 1.17–1.28 (m, 2H), 1.75–1.88 (m, 2H), 2.96 (s, 3H), 3.13 (s, 3H), 3.35–3.43 (m, 1H), 3.51 (d, 1H). ^{13}C NMR (D_2O, 75 MHz): δ 30.3, 32.8, 54.9, 56.2, 59.9, 71.6, 118.7, 170.7, 178.7, 180.1.

23. Compound **22** (3.07 g, 12.7 mmol) was dissolved in H_2O (20 mL) and cooled to 10°C. 1N NaOH (38.1 mL, 38.1 mmol) was added dropwise over 0.5 h and the resulting mixture stirred at 10–15°C for 6 h. The reaction mixture was concentrated in vacuo to a thick oil (4.25 g). The crude material was slurried in MeOH (75 mL) for 4 h at room temperature, filtered, and the resulting white solids were rinsed with MeOH (25 mL). Vacuum drying afforded 3.02 g of methanolate solvate of **23** (93.9%). ^{1}H NMR (D_2O, 300 MHz): δ 1.65–1.78 (m, 2H), 2.20–2.43 (m, 2H), 3.35 (s, 3H), 3.40–3.89 (d, 1H), 3.78–3.88 (m, 1H). ^{13}C NMR (D_2O, 75 MHz): δ 185.7, 181.6, 75.5, 63.1, 51.5, 36.9, 30.9.

24. Compound **23** (1.78 g, 7.0 mmol) was dissolved in 1N HCl (21.0 mL, 21 mmol) and heated at reflux for 7 h. The mixture was concentrated in vacuo to afford 2.3 g of a thick oil. The crude material was slurried in 10:1 acetone/H_2O (25 mL), filtered, and dried in vacuo to afford 1.28 g

(93.4%) of a white solid. ^{1}H NMR (D_2O, 300 MHz): δ 2.20–2.49 (m, 2H), 2.67–2.78 (m, 2H), 4.35–4.37 (d, 1H), 5.08–5.14 (m, 1H),

37b. Anal. Calcd for $C_{14}H_{17}NO_7$: C 54.02, H 5.50, N 4.50. Found C 57.47, H 5.15, N 4.74; ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.45–1.75 (m, 2H), 2.10–2.40 (m, 2H), 3.60–3.70 (m, 1H), 3.95 (dd, 1H), 5.00 (s, 2H), 7.23–7.50 (m, 5H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 28.3, 30.2, 59.3, 65.5, 69.7, 127.6, 127.7, 128.3, 136.9, 156.0, 171.9, 174.4.

37a. Anal. Calcd for $C_{14}H_{17}NO_7$: C 54.02, H 5.50, N 4.50. Found C 53.97, H 5.51, N 4.51. ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.60–1.80 (m, 2H), 2.20–2.43 (m, 2H), 3.65–3.82 (m, 1H), 4.35 (dd, 1H), 4.58 (s, 2H), 6.85–7.00 (m, 3H), 7.22–7.28 (m, 2H), 8.10 (d, 1H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 28.4, 30.2, 56.9, 66.6, 69.9, 114.6, 114.7, 121.1, 129.4, 157.6, 167.6, 171.3, 174.3.

39. Anal. Calcd for $C_{16}H_{22}N_2O_6$: C 56.80, H 6.55, N 8.28, O 28.37. Found: C 51.92, H 6.04, N 8.59, O 26.12; ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.60–1.80 (m, 2H), 2.20–2.50 (m, 2H), 2.80 (s, 3H), 2.95 (s, 3H), 3.65–3.80 (m, 1H), 4.0 (dd, ^{1}H), 5.05 (s, 2H), 7.28–7.40 (m, 5H), ^{13}C NMR (75 MHz, DMSO-d_6) δ 27.8, 28.6, 29.0, 334.0, 34.7, 36.6, 59.3, 65.4, 65.5, 70.1, 78.5, 127.6, 127.7, 128.3, 136.8, 136.9, 156.0, 172.0, 172.3.

40. Anal. Calcd for $C_{16}H_{23}N_3O_5$: C 56.96, H 6.87, N 12.45, O 23.71. Found C 56.59, H 7.05, N 11.62, O 21.86; ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.40–1.75 (m, 2H), 2.17–2.40 (m, 2H), 2.75 (s, 3H), 2.90 (s, 3H), 3.60 (m, 1H), 3.90 (t, 1H), 5.00 (s, 2H), 7.02 (s, 1H), 7.17 (d, 1H), 7.20–7.40 (m, 6H). ^{13}C NMR (75 MHz, DMSO-d_6) δ 28.5, 28.9, 34.7, 36.6, 59.3, 65.4, 70.1, 127.5, 127.7, 128.2, 137.0, 155.8, 172.1, 172.2.

42. Anal. Calcd for $C_{17}H_{25}N_3O_7S$: C 49.15, H 6.06, N 10.11, O 26.96, S 7.72. Found C 48.40, H 6.23, N 9.54, O 29.08, S 7.00; ^{1}H NMR (300 MHz, DMSO-d_6) δ 2.00–2.20 (m, 2H), 2.30 (s, 3H), 2.4 (t, 2H), 3.10 (s, 3H), 3.20 (s, 3H), 4.37 (dd, 1H), 5.05 (s, 2H), 5.50 (m, 1H), 7.20–7.40 (m, 6H), 7.60 (s, 1H), 7.80 (d, 1H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 23.94, 30.20, 34.23, 40.95, 56.29, 65.95, 91.03, 127.83, 128.28, 136.59, 156.50, 169.73, 179.89.

43. ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.85–2.00 (m, 1H), 2.05–2.20 (m, 1H), 2.30–2.60 (m, 4H), 4.17 (dd, 1H), 4.78 (dd, 1H), 5.05 (s, 2H), 7.20–7.50 (m, 7H), 7.65 (d, 1H).

55. A 100 mL three necked round-bottomed flask equipped with a magnetic stirring bar, addition funnel, thermometer and a positive nitrogen pressure was charged with 1 M *t*BuOK THF solution and anhydrous THF. To this at −78°C (dry ice/acetone bath) was added a solution of **54** dissolved in THF (10.0 mL) over a period of 4 min, while maintaining the temperature below −50°C for 75 min. The mixture was then quenched with trifluoroacetic acid (0.15 mL) and brought to room temperature. The reaction mixture was then concentrated to an off yellow precipitate. To the precipitate was then added methylene chloride (40 mL). The resulting precipitate was filtered and the filtrate concentrated to a yellow foam (120 mg, 60% yield). ^{1}H NMR (300 MHz DMSO-d_6) δ 1.64–1.77 (m, 2H), 2.38–2.45 (m, 2H), 3.65–3.72 (m, 1H), 4.57 (s, 2H), 5.13 (dd, 1H, J=4.7 Hz), 6.93–6.98 (m, 3H), 7.27–7.32 (m, 2H), 8.35 (s,1H), 8.89 (d,1H, J=8.9 Hz).

57. A solution of glycine (262.5 g, 3.5 mol), pyridoxal phosphate (0.92 g, 3.5 mmol), SHMT (35 g, ~0.35 mmol) in 3 L of water was prepared, the pH was adjusted to 7 with 5% ammonium hydroxide and then diluted to 3.5 L total volume with water. 4-Pentenal (81.6 g, 0.97 mol) was added over 5 h at 15°C and a pH of 6.9–7.1. After stirring an additional 2 h, the enzyme was removed from the reaction mixture by concentration on a 30,000 MW ultrafilter followed by diafilteration with 2.8 L of 0.01 M phosphate buffer (pH=6). The resulting product filtrate was evaporated under vacuum to a volume of ~5 L to remove unreacted 4-pentenal. Excess glycine was removed from the product solution which contained **57** (124 g) and the corresponding threo isomer (11 g), by adsorption onto 2.5 L of SP207 resin followed by washing with 1.5 L of water and elution with 5 L of 15% methanol. The SP207 eluent was concentrated under vacuum to a volume of 1.25 L. 2-Methoxyethanol (2.5 L) was added and the solution again concentrated to 1.25 L. The solid was filtered and washed with 2-methoxy ethanol (600 mL), and dried overnight at 60°C to yield the desired product (127 g, 82% yield, 93:7 *allo:threo* ratio). ^{1}H NMR (300 MHz DMSO-d_6) δ 1.50–1.80 (m, 2H), 2.00–2.40 (m, 2H), 3.92 (d, 1H), 4.00–4.20 (m, 1H), 5.00–5.20 (m, 2H), 5.80–6.00 (m, 1H).

58. A slurry of compound **57** (50 g, 0.314 mole, 8% *threo* isomer) in 600 mL of H_2O was generated in a 2 L beaker with a magnetic stirrer, a thermometer, a pH meter, and an addition funnel. Phenoxyacetyl chloride (47.78 mL, 1.10 equiv.) was added to the addition funnel. The amino acid slurry was cooled to 10°C and adjusted to pH 9.0–9.5 with 5N NaOH. The resulting solution was vigorously stirred while the acid chloride was added dropwise over 30 min, maintaining the pH at 9.0–9.5 and the temperature at 5–10°C. The reaction was stirred until the pH stabilized without the need for additional base. The reaction mixture was warmed to 25°C, then extracted with 250 mL of EtOAc, which was discarded (contains trace components and any unreacted acid chloride). A solution of 1 M H_2SO_4 was slowly added over 2 h to bring the pH to 2.0 (Note: seed the solution at the point when it turns cloudy, typically around pH 4.6). The product slurry was stirred for an additional h, and then filtered. The filter cake was rinsed with water and dried in a vacuum oven at 70°C to afford 84.6 g of **58** (HPLC: 97%, 4% *threo* isomer by ^{1}H NMR, 93% corrected yield). Anal. Calcd for $C_{14}H_{17}NO_7$: C 54.02, H 5.50, N 4.50. Found C 53.97, H 5.51, N 4.51; ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.60–1.80 (m, 2H), 2.20–2.43 (m, 2H), 3.65–3.82 (m, 1H), 4.35 (dd, 1H), 4.58 (s, 2H), 6.85–7.00 (m, 3H), 7.22–7.28 (m, 2H), 8.10 (d, 1H). ^{13}C NMR (75 MHz, DMSO) δ 28.4, 30.2, 56.9, 66.6, 69.9, 114.6, 114.7, 121.1, 129.4, 157.6, 167.6, 171.2, 174.3.

59. A solution of **58** (26.6 g; 90 mmol) in 300 mL of THF cooled to −50°C was treated with *N*-methylmorpholine (NMM, 10.2 mL, 92.8 mmol, 1 min), isobutylchloroformate (IBCF, 12.4 mL, 95.6 mmol, 1.05 equiv., 25 min), and cold ammonia (21 mL, 28%). The cooling bath was removed

from the rapidly stirring slurry (exotherm observed at 25°C) which was allowed to warm to room temperature. After concentrating under vacuum to a 95 g slurry, 300 mL of H_2O were added and the thick slurry was stirred for an additional 2 h. Filtration, rinsing, and drying at 40°C for two days yielded 22.7 g of **59** (HPLC: 99.7%; no *threo* isomer was present as indicated by ^{1}H NMR analysis; 95% corrected yield). Anal. Calcd for $C_{15}H_{20}N_2O_4$: C 61.63, H 6.90, N 9.58, O 21.89. Found C 61.45, H 6.98, N 9.48, O 21.91; ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.20–1.50 (m, 2H), 1.88–2.20 (m, 2H), 3.62 (m, 1H), 4.23 (dd, 1H), 4.55 (s, 2H), 4.80–5.02 (m, 3H), 5.65–5.80 (m, 1H), 6.80–7.00 (m, 3H), 7.10 (s, 1H), 7.25 (t, 2H), 7.40 (s, 1H), 7.83 (d, 1H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 29.4, 31.9, 56.8, 66.6, 70.1, 114.6, 121.1, 129.4, 138.6, 157.6, 167.4, 171.7, 204.3.

60. A slurry of **59** (20.0 g, 68 mmol) in 80 mL of CH_2Cl_2 and 66.1 mL of 2,6-lutidine (0.567 mmol, 8.3 equiv.) formed in a 2 L three-neck round-bottom flask equipped with a mechanical stirrer, a nitrogen bubbler, and a thermometer was stirred at room temperature. Methanesulfonyl chloride (6.88 mL, 89 mmol, 1.3 equiv.) was added to the slurry all at once, and the mixture was allowed to exotherm to room temperature. After 3 h, 500 mL of additional CH_2Cl_2 was added and the solution was placed into a separatory funnel. The organic solution was extracted with 2×250 mL of 1.5 M H_3PO_4, then concentrated to 60 g of a thick slurry, which was triturated with 250 mL of Et_2O and 15 mL of MeOH, stirred for 2 h, and then filtered. The filter cake was rinsed well with Et_2O, and dried at 43°C in a vacuum oven overnight to give 21.6 g of **60** (HPLC: 99.3%, corrected yield 88.2%): Anal. Calcd for $C_{16}H_{22}N_2O_6S$: C 51.88, H 5.99, N 7.56, O 25.92, S 8.66. Found C 50.80, H 5.88, N7.23, O 23.91, S 8.53; ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.52–1.75 (m, 2H), 1.90–2.20 (m, 2H), 3.10 (s, 3H), 4.58 (s, 2H), 4.78 (dd, 1H), 4.85 (m, 1H), 4.90–5.06 (m, 2H), 4.75 (m, 1H), 6.82–6.95 (m, 3H), 7.25 (t, 2H), 7.40 (s, 1H), 7.75 (s, 1H), 8.27 (d, 1H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 28.5, 29.0, 38.0, 53.8, 66.36, 80.6, 114.4, 114.5, 115.4, 121.0, 129.4, 137.2, 157.6, 168.0, 169.5.

62. To a solution of 2,6-lutidine (2.33 mL, 20 mmol, 7.4 equiv.) in 15 mL of CH_2Cl_2 was added $ClSO_3H$ (0.66 mL, 10 mmol, 3.7 equiv.) over 20 min at −12°C. The cold mixture was stirred for 30 min then 1.00 g of **60** (HPLC: 98%, 27 mmol) was added all at once. The cold reaction mixture was stirred for 5 h at −10 to −15°C, then placed in the freezer at −25°C overnight. The resulting cold slurry was added to 15 g of ice water and kept cold while monitoring the pH. After 1 h, the pH had stabilized at around 1.0. The two-phase mixture was placed into a pre-chilled separatory funnel and the layers separated. The aqueous layer was extracted with 2×2 mL of CH_2Cl_2, and the combined organic layers were placed in the freezer (or it could be carried directly into the next step of β-lactam ring closure). This solution was found to be stable for at least one week. Exact yields using this process were difficult to determine since the product was not isolated but left as a solution and carried on into the next step. However, based on HPLC analyses and yields typically obtained in the β-lactam closure, an estimated sulfamation yield of 95% was obtained. An analytical sample of its tetrabutylammonium salt was made and purified: Anal. Calcd for $C_{16}H_{36}N$. $C_{16}H_{21}N_2O_9S_2$: C 55.55, H 8.30, N 6.07, O 20.81, S 9.27. Found C 55.54, H 8.09, N 5.96, O 20.83, S 9.01; ^{1}H NMR (300 MHz, DMSO-d_6) δ 0.80 (t, 12H), 1.23 (q, 8H), 1.50 (m, 10H), 1.90–2.20 (m, 2H), 2.95–3.20 (m, 11H), 4.55 (s, 2H), 4.75 (m, 2H), 4.95 (m, 2H), 5.70 (m, 1H), 6.80–7.00 (m, 3H), 7.24 (t, 2H), 8.20 (d, 2H), 10.23 (s, 1H).

63. Three-step (mesylation, sulfamation, and β-lactam closure), one-pot procedure: A slurry of **59** (1 g, 34 mmol) in 4 mL of CH_2Cl_2 and 3.80 mL of 2,6-lutidine (32.5 mmol, 9.5 equiv.) was generated in a 25 mL, 3-neck round-bottom flask equipped with a magnetic stirrer, a nitrogen bubbler, a thermometer, and an addition funnel containing 0.40 mL of MsCl (50 mmol, 1.5 equiv.), and another addition funnel containing 0.93 mL of $ClSO_3H$ (140 mmol, 4.1 equiv.). The MsCl was added over 5 min and the mixture stirred for 2.5 h. The resulting slurry was cooled to −30°C, then the $ClSO_3H$ was very carefully added over 15 min, keeping the temperature between −16 to −20°C during the addition. The thick slurry was allowed to warm to −6 to −10°C, stirred for 2 h, then placed into the freezer at −25°C and left overnight. An HPLC profile of the reaction indicated 94.1% of **62** and 1% of **60**. The thick slurry was added to 10 g ice water and stirred at 0–3°C for 35 min (resulting in a pH of 0.95), then placed into a pre-chilled separatory funnel and the layers separated. The aqueous layer was extracted with 2×2 mL of CH_2Cl_2 and the organic layers combined. Water (10 mL) was added and the biphasic mixture was concentrated under vacuum to give 11 g of a solution, while keeping the mixture cold at all times (a room temperature water bath was used). The cold solution (pH=2.34) was poured into a beaker and 1.9 mL of 5 N KOH was added until the pH reached 10.0 (a solid precipitated at pH 8.8). The cold bath was removed and the reaction mixture was allowed to warm to room temperature, and the pH adjusted to 10 with 5N KOH. A thick slurry formed upon stirring for 2.5 h. After adjustment with conc. HCl to a pH of 5.6, the mixture was cooled with an ice bath and 5 mL of saturated KCl was added. The slurry was stirred for 1 h, filtered, and dried under vacuum at 45°C overnight to give 1.02 g of compound **63** as a monohydrate (HPLC: 94%, corrected yield=68.5% over three steps for an average of 88.2%/step): Anal. Calcd for $C_{15}H_{19}N_2O_7S$: C 45.90, H 4.37, N 7.14, S 8.27. Found C 45.68, H 4.40, N 6.73, S 8.27.

64. Compound **63** (10 g, 24.4 mmol) and 100 mL of THF was stirred and cooled to <5°C. To this was added 0.10 mL of conc. HCl (1.2 mmol; ~0.05 equiv.). The mixture was allowed to warm to 5–7°C and held at this temperature range for 1 h. HPLC analysis showed 0.75% of the starting material remained. The reaction was quenched after 70 min by adding 100 mL of water. The pH of the quenched reaction was 1.6. THF was evaporated and the resulting crystallized product was filtered and washed with water, dried under vacuum at room temperature to give 6.22 g of a nearly white solid (96.9% yield): Anal. Calcd for $C_{15}H_{18}N_2O_3$: C 65.68, H 6.61, N 10.21. Found C 65.93, H 6.56, N 10.33. ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.35–1.60 (m, 2H), 1.82–2.10 (m, 2H), 3.63 (m, 1H), 4.58 (s, 2H), 4.90–5.08 (m, 2H),

5.12 (m, 1H), 5.80 (m, 1H), 6.90–7.00 (m, 3H), 7.30 (t, 2H), 8.40 (s, 1H), 8.90 (d, 1H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 29.3, 29.6, 52.7, 57.6, 66.5, 114.5, 115.0, 121.0, 129.3, 137.8, 157.7, 167.0, 168.1.

65. Compound **64** (10 g, 36.5 mmol) was stirred with 100 mL of DMF while 5.18 mL (54.8 mmol) of methyl bromoacetate was added. This was followed by addition of 8.58 g (62.1 mmol) of powered K_2CO_3 and the reaction mixture was stirred at room temperature for 48 h when HPLC analysis showed that the alkylation reaction was complete. The reaction mixture was diluted with 100 mL of CH_2Cl_2 and washed with 5×100 mL of 1N HCl to remove the DMF. The organic layer was stirred with 100 mL of H_2O, 1.77 g (5.48 mmol) of Bu_4NBr follwed by 5N NaOH to adjust the pH to 13. After having been stirred for 5 h, the layers were separated, and an equal volume of CH_2Cl_2 was added to the aqueous layer. The pH was lowered to 6.8 with 5N HCl and 8.67 g (40.2 mmol) of *p*-nitrobenzyl bromide was added. The mixture was stirred at room temperature for 12 h and. the layers separated. The organic layer was washed with 100 mL of H_2O and evaporated. The resulting solid was slurried in 375 mL of 80:20 MTBE/hexane for 1 h, filtered and washed sequentially with 100 mL of methyl-*t*-butyl-ether and 100 mL of isopropanol to give compound **65** in 48% yield. Anal. Calcd for $C_{24}H_{25}N_3O_7$: C 61.66, H 3.37, N 8.99. Found C 61.58, H 3.61, N 8.87. ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.40–1.60 (m, 1H), 1.60–1.90 (m, 3H), 3.78 (m, 1H), 4.20 (q, 2H), 4.50 (s, 2H), 4.80–5.00 (m 2H), 5.15 (dd, 1H), 5.30 (s, 2H), 5.67 (m, 1H), 6.80–7.00 (m, 3H), 7.23 (t, 2H), 7.62 (d, 2H), 8.20 (d, 2H), 9.00 (d, 1H).

66. Compound **65** (0.92 g, 2 mmol) was charged under a nitrogen purge into a 100 mL, 3-neck flask equipped with a thermometer, a subsurface gas inlet, and a gas outlet. To this was added 20 mL of dry acetonitrile, 2 mL of H_2O (10% of volume) and 1 mL of a 0.05 wt% solution of Sudan IIIB dye which turned the solution red. The solution was cooled to −10°C and ozone was slowly introduced. The red color began to lighten slowly, and after 2 min, into a slightly yellow one, indicating the starting material had been converted into ozonide intermediates. The flask was removed from the cold bath and was treated with 0.68 mL (6 mmol) of 30% H_2O_2. This solution was added into a separate flask containing a pH 4 buffer solution made from 7 mL of H_2O and 0.64 g of NaH_2PO_4 and heated to 55°C. A solution of NaO_2Cl (80%, 0.40 g, 3.5 mmol) in 2.8 mL of H_2O was added dropwise into the reaction mixture over 2 h via a syringe pump. The reaction mixture was cooled to room temperature, treated with 0.2 g of Na_2SO_3 to quench excess oxidant, and then acidified with concentrated HCl to pH 1. Acetonitrile was evaporated and 2 mL of Et_2O was added to crystallize the product, which was obtained in 82% yield after being filtered, washed sequentially with H_2O and Et_2O and dried in vacuo: Anal. Calcd for $C_{16}H_{20}N_2O_5$: C 56.91, H 4.78, N 8.66. Found C 57.11, H 5.00, N 8.60; ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.55–1.80 (m, 1H), 1.83–2.00 (m, 1H), 2.10 (t, 2H), 3.80 (m, 1H), 4.20 (q, 2H), 4.58 (s, 2H), 5.12 (dd, 1H), 5.30 (s, 2H), 6.80–7.00 (m, 2H), 7.23 (t, 2H), 7.62 (d, 2H), 8.20 (d, 2H), 9.03 (d, 1H).

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