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Published on Web 06/18/2003

Carbapenem Biosynthesis: Confirmation of Stereochemical Assignments and the Role of CarC in the Ring Stereoinversion Process from L-Proline

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Abstract: (5*R*)-Carbapen-2-em-3-carboxylic acid is the simplest structurally among the naturally occurring carbapenem β-lactam antibiotics. It co-occurs with two saturated (3*S*,5*S*)- and (3*S*,5*R*)-carbapenam carboxylic acids. Confusion persists in the literature about the signs of rotation and absolute configurations of these compounds that is resolved in this paper. (3*S*,5*S*)-Carbapenam carboxylic acid was prepared from L-pyroglutamic acid to unambiguously establish its absolute configuration as identical to the natural product isolated from *Serratia marcescens* and from overexpression of the biosynthetic genes *carAB* in *Escherichia coli*. L-Proline labeled with deuterium or tritium at the diastereotopic C-5 methylene loci was shown to incorporate one label at the bridgehead of (3*S*,5*S*)-carbapenam carboxylic acid, but not into the "inverted" (3*S*,5*R*)-carbapenam carboxylic acid or the final carbapenem product. CarC, the third enzyme of the biosynthetic pathway required to synthesize the carbapenem, was demonstrated in cell-free studies to be dependent on α-ketoglutarate and ascorbate in keeping with weak sequence identities with other non-heme iron, α-ketoglutarate-dependent oxygenases. CarC mediated the stereoinversion of synthetic (3*S*,5*S*)-carbapenam carboxylic acid to the (5*R*)-carbapenem as judged by bioassay. These findings suggest that L-proline is desaturated to pyrroline-5-carboxylic acid prior to uptake into the biosynthetic pathway. The loss of the bridgehead hydrogen from the (3*S*,5*S*)-carbapenam during the ring inversion process to form the epimeric (3*S*,5*R*)-carbapenam and desaturation to the (5*R*)-carbapenem are proposed to be coupled by CarC to the reduction of dioxygen to drive the formation of these higher energy products, an unprecedented reaction for this enzyme class.

Carbapenem-3-carboxylic acid (**4**) is the simplest of over 50 naturally occurring carbapenem β-lactam antibiotics.[1] Members of this family and their derivatives are clinically important for their potent, broad-spectrum antibacterial activity and their resistance to β-lactamases.[2] Early experiments established that the β-lactam carbons of **4** are derived from acetate[3] and the fused pyrrolidine carboxylic acid arises ultimately from glutamate.[3] Assembly of its primary metabolic precursors into the carbapenem nucleus takes place through the action of just three enzymes, CarA, B, and C (Scheme 1).[4] This efficient biosynthetic process is accompanied by a remarkable stereochemical inversion at C-5 mediated by CarC.[4] While the absolute configurations of **2**, **3**, and **4** were in part misassigned initially,[5] stereochemical correlations by Bycroft corrected these to those shown in Scheme 1.[6] A chemical logic can be discerned in this pathway where a natural L-amino acid is utilized in the formation of **1** by CarB and cyclized to carbapenam **2** by CarA. CarC then acts to invert the absolute configuration of the bridgehead to 5*R* essential to the ultimate biological activity of the antibiotic and to subtly raise the strain energy of the bicyclic system by placing the C-3 carboxyl *endo*.[4]

Unfortunately, the amended stereochemical assignments of Bycroft, which we relied upon in the interpretation of the biochemical experiments summarized in Scheme 1,[4] have been called into question by Ogasawara et al.,[7] and are further clouded by reversal of the absolute stereochemistry of **2** to (3*R*,5*R*) in a 1998 review.[8] We describe in this paper a third stereochemical correlation in which **2** as its *p*-nitrobenzyl (PNB) ester was prepared by total synthesis from L-pyroglutamic acid and compared to the same product isolated from wild-type *Serratia marcescens* and a recombinant *Escherichia coli* bearing *carAB* in an overexpression vector. These findings unambiguously confirm the corrected absolute configurations made for **2**, **3**, and **4** by Bycroft as depicted in Scheme 1.[4]

In addition, we address the issue of the cryptic stereochemical inversion at C-5 that occurs in the CarC-catalyzed transformation

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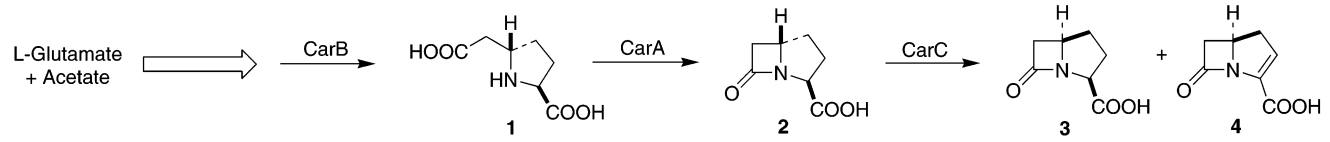
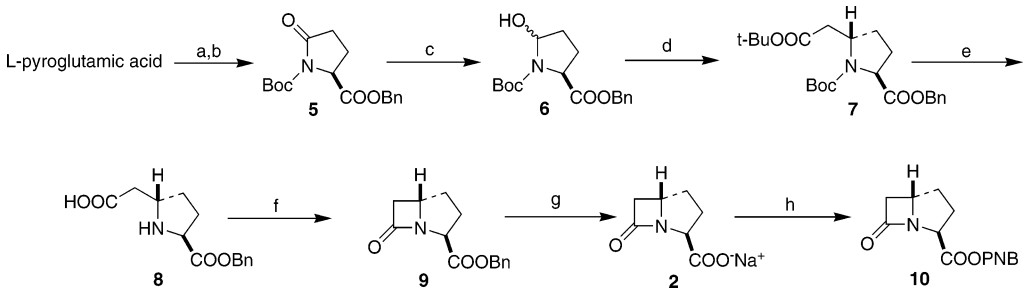
10.1021/ja034248a CCC: $25.00

Scheme 1



Scheme 2 [a]



[a] Reagents and conditions: (a) benzyl bromide, CH_2Cl_2, DIEA, 24 h; (b) $(Boc)_2O$, DMAP, triethylamine, CH_3CN, 18 h; (c) Superhydride, THF, −78 °C, 20 min; (d) *tert*-butyl diethylphosphonoacetate, potassium hydride, DMF, 1 h; (e) trifluoroacetic acid, 30 min; (f) tris(2,3-dihydro-2-oxobenzoxazol-3-yl)phosphine oxide (refs 11, 12), triethylamine, CH_3CN, 6 h; (g) Pd/C, H_2, EtOAc, sodium bicarbonate, 15 psi, 90 min; (h) *p*-nitrobenzylbromide, DMF, 4 h.

of **2** to **3** and **4**. L-Proline bearing first deuterium and then tritium at C-5 was incorporated into **2**, **3**, and **4** by *S. marcescens*. By both NMR spectroscopic means and double radioisotope analysis, we have been able to demonstrate that one isotopic label is retained at the bridgehead position in (3*S*,5*S*)-carbapenam **2**, but is cleanly lost in the epimerized (3*S*,5*R*)-carbapenam **3** and its carbapenem partner **4**. Apart from revealing the probable intermediacy of pyrroline-5-carboxylate/glutamyl semialdehyde as the immediate precursor from primary metabolism in carbapenam/em formation, the outcome of these experiments limits the possible mechanisms that can be acting in the ring inversion process.

Results and Discussion

Stereochemical Analysis. The potent antibiotic activity of the carbapenem was taken initially to establish the (5*R*)-configuration at the ring junction. Catalytic hydrogenation of **4** gave a 9:1 mixture of carbapenams **3** and (in fact) the enantiomer of **2**, respectively.[5] An insufficient amount of the reactive **2** was obtained as its *p*-nitrobenzyl (PNB) ester to allow accurate characterization of its absolute configuration. This ambiguity was remedied in a subsequent stereochemical correlation in which the antipode of **2** was prepared from D-glutamic acid and found to be identical spectroscopically, except it displayed a CD spectrum equal and opposite to that of the natural product, thus establishing the true absolute stereostructure of **2** as (3*S*,5*S*) shown in Scheme 1.[6]

Unfortunately, while the conclusions of the second Bycroft paper were correct, as we confirm below, clerical errors in the reported signs of optical rotations have led to a second stereochemical correlation described recently by Ogasawara et al.[7] Here 3-hydroxytetrahydropyridine of known absolute stereochemistry was converted to the enantiomer of **2** and, based on its optical rotation, the absolute configuration revised to Bycroft's original assignment.[5] We have independently reexamined this question and have prepared **2** by unambiguous total synthesis from L-pyroglutamic acid and compared the optical rotation of its PNB ester to that of both the natural product isolated from *S. marcescens* and the corresponding metabolite generated by an overexpression clone of *carAB* in *E. coli*. All three were found to have the same absolute configuration.

Stereochemical Correlation of (3*S*,5*S*)−Carbapenam 2. Synthesis of the PNB ester **10** of **2** employed the Horner−Emmons−Smith reaction used previously to prepare protected carboxymethylprolines.[9] As shown in Scheme 2, *N*-Boc benzyl L-pyroglutamate **5**[10] was reduced to the hemiaminal **6** by treatment with Superhydride.[11] Coupling with *tert*-butyl diethylphosphonoacetate gave protected (2*S*,5*S*)-carboxymethylproline **7** in a diastereomeric excess of 7:1, in accord with the literature as determined by ^{1}H NMR spectroscopy.[9] The mixture of diastereomers was treated with TFA to yield 5-carboxymethyl proline α-benzyl ester **8**. Cyclization of the protected β-amino acids with a particularly efficient peptide coupling reagent[12,13] formed the β-lactam ring. Separation from the minor diastereomer by silica gel chromatography yielded the benzyl ester of the (3*S*,5*S*)-carbapenam **9** in 25% overall yield for 5 steps. Hydrogenolysis of the benzyl ester in the presence of 1 equiv of $NaHCO_3$ yielded the carbapenam sodium salt **2** in nearly pure form, accompanied by varying amounts of the hydrolysis product **1** as a contaminant (≤10%). Carbapenam **2** is surprisingly stable in neutral solution, but is prone to hydrolysis on exposure to protic/Bronsted acids, base, or divalent cations. Losses were also observed upon lyophilization/concentration after HPLC during attempts to further purify **2** (unpublished results).

To complete the stereochemical correlations, **2** was treated with *p*-nitrobenzyl bromide in DMF to yield the PNB ester **10**, which was used for comparison to the carbapenams isolated from *S. marcescens* as well as the single carbapenam produced by the *E. coli carAB* transformant described previously.[4] Samples of the PNB esters from the three sources showed identical ^{1}H NMR spectra and HPLC/TLC chromatographic

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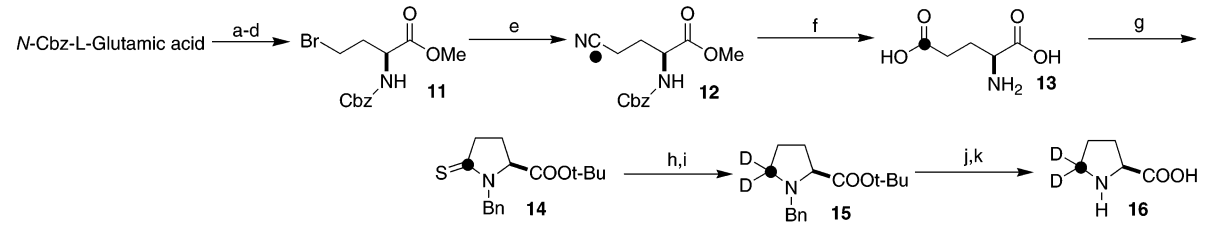
Scheme 3 [a]



[a] (a) $(CH_2O)_n$, TsOH, toluene, 5 h; (b) 0.1 N NaOMe/MeOH, 18 h; (c) isobutyl chloroformate, triethylamine, *N*-hydroxy-2-thiopyridone, 1 h; (d) bromotrichloromethane, 3 h; (e) sodium [^{13}C]cyanide, DMSO, 3 h; (f) 2:1 acetic acid/HCl, 6 h; (g) ref 23; (h) methyl iodide, CH_3CN, 18 h; (i) sodium borodeuteride, MeOH, 3 h; (j) trifluoroacetic acid; (k) $Pd(OH)_2$, H_2, EtOH, 2 d.

properties. Most important, optical rotation data for the PNB esters isolated from the three sources were nearly identical (total synthesis $[\alpha]^{25}_D$ −168.2°, *S. marcescens* $[\alpha]^{25}_D$ −173.0°, *E. coli carAB* $[\alpha]^{25}_D$ −169.6°).

On the basis of these findings, the absolute configuration of **2** is indeed (3*S*,5*S*) as determined earlier by Bycroft.[6] The data collected indicate, however, that Bycroft incorrectly reported the sign of the optical rotation for the methyl ester antipode of **2** prepared in his study. Nevertheless, his assignment of the (3*S*,5*S*) stereochemistry for **2** isolated from the natural source was, indeed, correct.

Carbapenam(em) Stereoinversion. With the absolute configurations of **2**, **3**, and **4** firmly in hand, attention was turned to the striking ring inversion process carried out by CarC (Scheme 1).[4] Primary sequence alignment of CarC with other known proteins revealed a modest 23%[14] identity to clavaminate synthase (CS2)[15] and weak identities to other α-ketoglutarate-dependent dioxygenases. These Fe(II)-requiring enzymes are commonly associated with hydroxylation reactions at unactivated carbon centers and are also known to mediate desaturation reactions and oxidative cyclization processes.[16−18] Therefore, while introduction of the double bond in **4** is unsurprising, the ring inversion involving no change in oxidation state in the formation of **3** has no precedent that we are aware of among the reactions catalyzed by this class of enzymes.

As a first step toward understanding this ring stereoinversion process, we undertook an examination of the fate of the bridgehead hydrogen at C-5 in **2** into **3** and **4**. Previous incorporation experiments had demonstrated that the pyrrolidine ring is derived from L-glutamate.[3] We reasoned, on the other hand, that the more direct precursor from primary metabolism could be L-proline on the basis of a downstream gene in the biosynthetic cluster that appears to encode a proline dehydrogenase.[14] Whether or not hydrogen isotope remained at the C-5 position in the bicyclic products can be visualized to provide information about the assembly of **2**, **3**, and **4** from their true primary metabolic precursors. If [5,5-$^2H_2$,5-^{13}C]-L-proline were oxidized to an L-glutamate equivalent prior to the assembly of **2**, **3**, or **4**, isotopic label would be lost and there would be no spin−spin coupling resulting from a directly bonded deuterium atom detectable by ^{13}C NMR spectroscopy. Conversely, if oxidation of labeled L-proline did not proceed beyond the pyrroline-5-carboxylate/glutamyl semialdehyde stage, one deuterium would be retained at C-5 detectable as a 1:1:1 triplet by ^{13}C NMR, and potentially detectable in all three β-lactam compounds.

[5,5-$^2H_2$,5-^{13}C]-L-Proline. As outlined in Scheme 3, *N*-Cbz L-glutamate was converted to its α-methyl ester according to the procedure of Hanessian.[19,20] Using the Barton decarboxylative halogenation method,[21] the remaining carboxyl was converted to the terminal bromide **11** by photolysis of the intermediate *N*-hydroxy-2-thiopyridone ester in the presence of bromotrichloromethane.[22] The bromide was then displaced by NaCN in DMSO to provide **12**. Hydrolysis of the nitrile in 2:1 acetic acid/concentrated HCl[23] and recrystallization from pyridine/ethanol provided L-glutamic acid **13** in pure form. Following established procedures,[24] L-glutamate was then *N*-benzylated, cyclized to its *N*-benzyl pyroglutamate, protected as *tert*-butyl ester, and converted to the thiolactam **14**. Reaction of **14** with 10 equiv of methyl iodide in acetonitrile, followed by reduction with sodium borohydride,[25] yielded *tert*-butyl *N*-benzyl-L-proline **15**. The fully protected amino acid was then treated with TFA followed by catalytic hydrogenation to yield L-proline **16**.

This sequence was repeated using sodium [^{13}C]cyanide to displace bromide **11** and sodium borodeuteride to reduce the [5-^{13}C]thiolactam **14**. Deprotection of the triply labeled **15** yielded [5,5-$^2H_2$,5-^{13}C]-L-proline in 12% overall yield from [5-^{13}C]-L-glutamic acid having 85−90% deuterium/site as determined by ^{1}H NMR and mass spectrometric analysis.

The multiply labeled L-proline was administered to a *S. marcescens* fermentation, and the β-lactams **2**, **3**, and **4** produced were extracted, derivatized as their PNB esters, and purified[3] for ^{13}C NMR analysis. The ^{13}C spectra showed that one deuterium was retained at the C-5 position of **2**, J_{C-D} = 23.3 Hz, whereas no deuterium was detectable at the corresponding locus in **3** or **4**. Incorporation levels of [5,5-$^2H_2$,5-^{13}C]-L-proline into the β-lactam products were low but discernible, presumably owing to kinetic isotope effects occurring during reactions at this center, and the inherently inefficient spin relaxation of the [^{13}C-^{2}H] labeled species suppressing signal intensities. A more

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Table 1. Double Isotope Analysis of [5-^{3}H,U-^{14}C]-L-Proline Incorporation into **2**, **3**, and **4**

	^{3}H/^{14}C	^{3}H retention (%)
[5-^{3}H,U-^{14}C]-L-proline	10.38	
[5-^{3}H,^{14}C]-**2**	7.78	75
[5-^{3}H,^{14}C]-**3**	0.14	1
[5-^{3}H,^{14}C]-**4**	0.32	3

sensitive means to detect the retention or loss of bridgehead label was sought to ensure the validity of this result.

Commercially available [U-^{14}C]-L-proline and [5-^{3}H]-L-proline were combined to achieve a ^{3}H/^{14}C ratio of 10.38. The doubly labeled amino acid was added to a growing fermentation of *S. marcescens* and the β-lactams produced were isolated, derivatized, and purified by HPLC. The results of the radioisotope analysis are presented in Table 1. Carbapenam **2** retained a high proportion of tritium label relative to the ^{14}C internal standard, 75%. In contrast, inversion of the ring junction that occurs in the formation of **3** and **4** was accompanied by 99% and 97% loss of tritium, respectively. These more quantitative findings are in complete accord with the observations made above by ^{13}C NMR spectroscopy. The biosynthetic path to the pyrrolidine ring in **2**, **3**, and **4**, therefore, proceeds by way of the intermediate oxidation state in pyrroline-5-carboxylate/glutamyl semialdehyde **17**, presumably by the oxidation of L-proline.

Preliminary Cell-Free Conversion of 2 to 3 and 4 by CarC. The separate expressions of *carAB* and *carABC* in *E. coli* gave[4] the unexpected result that (3*S*,5*S*)-carbapenam **2** precedes the formation of the "inverted" carbapenam **3** and its unsaturated partner **4** and implies that this stereoinversion is performed by CarC. To gather further support for this remarkable observation, the availability of enantiomerically pure, synthetic **2** made possible a direct test of its conversion to **3** and **4** by a cell-free extract containing recombinant CarC. Overexpression of *carC* was carried out in pET24a and BL21(DE3) as host under standard conditions. The cells were collected by centrifugation, suspended in phosphate buffer and glycerol, lysed in the presence of protease inhibitors, and treated with DNase and RNase, and the cell debris was removed by centrifugation. The clarified supernatant was taken as the cell-free extract (CFE).

The ability of the CFE to catalyze carbapenem synthesis was monitored in a paper disk assay. A β-lactam supersensitive *E. coli* (SC12155) was used as the test organism. As shown in Figure 1, incubation of carbapenam **2** with the CFE and added α-ketoglutarate and ascorbate gave a clear zone of inhibition. Controls with just the carbapenam itself, or with the complete incubation mixture but lacking **2**, failed to give zones of inhibition. Additional controls demonstrated antibiotic production was dependent on added α-ketoglutarate, reduced in the absence of added ascorbate, but was minimally affected by addition of ferrous ammonium sulfate (data not shown). These observations support the view from primary sequence similarities that CarC is an α-ketoglutarate-dependent, non-heme iron oxygenase and confirms the deduction made earlier that it mediates the oxidative conversion of **2** to carbapenem **4**.[4]

Conclusions

In this work we have established with certainty the absolute configuration of carbapenam **2** by total synthesis and confirmed the amended stereochemical assignments of **3** and **4** by Bycroft.[6]

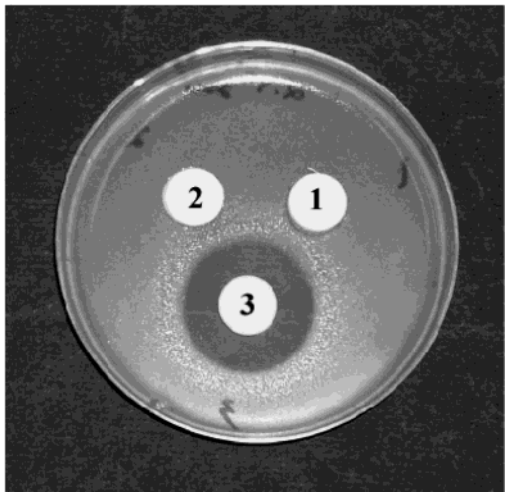

Figure 1. Presumed carbapenem **4** production in a cell-free extract (CFE) of pET24a/*carC* BL21(DE3) *E. coli* visualized on a plate of β-lactam supersensitive *E. coli* strain SC12155. (1) 2 mM carbapenam **2**, sodium salt (2) CarC CFE, 8 mM α-ketoglutarate, 1 mM ascorbate; (3) CarC CFE, 2 mM carbapenam **2**, sodium salt, 8 mM α-ketoglutarate, 1 mM ascorbate.

To distinguish the biochemical origins of the pyrrolidine ring of these natural products, incorporation experiments with [5,5-^{2}H$_2$,5-^{13}C]- and of [5-^{3}H,U-^{14}C]-L-proline were conducted in growing cultures of *S. marcescens*. Analysis of the PNB esters of **2**, **3**, and **4** first by ^{13}C NMR spectroscopy and second by two-channel scintillation counting demonstrated retention of deuterium or tritium label at the bridgehead carbon in **2**, but its complete loss on ring inversion to **3** and **4**. The paired radioisotope analysis revealed a 75% retention of tritium label in **2** from L-proline. Unfortunately, the distribution of radioisotope between the two diastereotopic C-5 L-proline positions is not known. The high extent of tritium retention may be due to unequal distribution of label at the C-5 loci of L-proline and/or a substantial kinetic isotope effect in the stereochemical inversion process mediated by CarC. Primary sequence alignment of CarC shows weak similarity to α-ketoglutarate-dependent, non-heme iron oxygenases,[14] suggesting that this unusual process may be fundamentally oxidative. In the event, the double-labeled proline experiments support the view that the primary metabolic precursor of (2*S*,5*S*)-carboxymethylproline (**1**), the first committed intermediate of carbapenem biosynthesis, is pyrroline-5-carboxylate/glutamyl semialdehyde (**17**, Scheme 4) derived from L-proline.

Preliminary cell-free experiments with CarC overproduced in *E. coli* demonstrate apparent carbapenem **4** production by bioassay against a β-lactam sensitive test organism. As anticipated from sequence alignments, added α-ketoglutarate and ascorbate enlarged the zones of growth inhibition in the bioassay. Experiments to purify CarC are underway to examine the unprecedented ring inversion process and attendant desaturation. Curiously, in the unrelated biosynthesis of the potent β-lactamase inhibitor clavulanic acid **20** an unknown, but also likely oxidative process,[26] inverts clavaminic acid **18** to the aldehyde **19**, which is reduced to **20**.[27] In this overall conversion,

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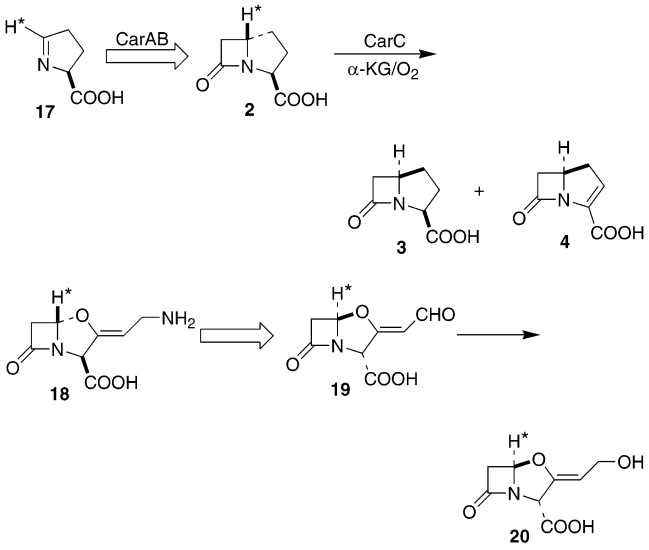
Scheme 4



however, the bridgehead hydrogen is retained.[28] While neither of these oxidative "enantiomerizations" is understood, both reflect a biosynthetic strategy to create these powerfully bioactive substances initially in their inactive, "enantiomeric" forms—which are carried through many steps in the case of clavulanic acid—only to reveal each in its true colors through a penultimate oxidative ring inversion step. The means by which this stereochemical change is accomplished without apparent alteration of oxidation state in carbapenam **3** is an intriguing problem. The formation of **3** from **2**, however, is in itself an uphill event thermodynamically (C-3 carboxylate becomes *endo*) and one may speculate that loss of H-5 from **2** and its replacement in **3** from another source is somehow coupled with reduction of dioxygen to drive the overall reaction. How this process is achieved is the object of current investigation.

Note Added in Proof. In a recent communication Bycroft corrects his inadvertently misreported optical rotation of (3*R*,5*R*)-carbapenam methyl ester.[29] Unfortunately, this error has been propagated again in a total synthesis of all four stereoisomers of this compound.[30]

Experimental Section

Tetrahydrofuran was distilled from sodium/benzophenone ketyl; acetonitrile was distilled from CaH_2. Reagents were obtained from Aldrich and used as received unless otherwise noted. Sodium [^{13}C]-cyanide (99%) and sodium borodeuteride (99%) were purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA). Melting points are uncorrected. Optical rotations were recorded on a Jasco Model P-1010 polarimeter. Scintillation analysis was performed on a Beckman Coulter Model LS 5801 liquid scintillation counter. Flash chromatography was performed with Bodman Industries silica gel 60 (230−400 mesh ATM). NMR spectral data were obtained on a Varian Unity^{Plus} 400 spectrometer, unless otherwise noted. NMR spectra recorded in $CDCl_3$ are reported in parts per million (δ) downfield from Me_4Si (^{1}H) or relative to $CDCl_3$ at 77.0 ppm (^{13}C). NMR spectra recorded in D_2O are reported in parts per million (δ) relative from D_2O 4.80 ppm (^{1}H) or relative to dioxane at 66.0 ppm (^{13}C).

Media used for the *S. marcescens* feeding experiments and the isolation, derivatization, and purification of PNB-protected β-lactams were performed as previously described.[4] [5,5-^{2}H$_2$,5-^{13}C]-L-Proline was administered to a 3 L *S. marcescens* fermentation at 1.3 mM total concentration. Radiolabeled [U-^{14}C]-L-proline and [5-^{3}H]-L-proline were purchased from Amersham Biosciences (Piscataway, NJ), mixed with cold L-proline (114 mg, 1 mmol), passed through a cation exchange column (Bond Elut, SCX, Varian, Walnut Creek, CA), and lyophilized. A portion was purified by HPLC and the ^{3}H/^{14}C ratio (10.38) determined prior to administration. Carbapenem production is analyzed by the disk diffusion method with β-lactam supersensitive *E. coli* SC12155 (obtained from Bristol Myers Squibb Inc., Princeton, NJ).

***β*-Lactam Production and Isotopic Incorporation Experiments: S. marcescens.** *S. marcescens* (ATCC 39006) fermentation was modified from the method of Bycroft et al.[3] Seed medium (100 mL, Bacto-Soytone 2%, sucrose 0.2%) was inoculated with a single colony maintained on Nutrient Agar (Becton Dickinson, Sparks, MD). After growing at 26 °C for 20 h with shaking at 300 rpm, 75 mL was used to inoculate 3 L of fermentation medium[3] in a MBF-500 bioreactor (Wheaton Instruments, Willville, NJ). For the radiolabeled L-proline incorporation experiment, the scale of the fermentation was 1 L and all other conditions remained the same. The fermentation was carried out for 26 h at 26 °C with 300 rpm stirring and 4 NI/min aeration. For both incorporation experiments, the L-prolines were added at the 4th hour of the fermentation. The pH was monitored and controlled to 7.5. Cells were separated by centrifugation at 4000*g* for 10 min at 4 °C.

Genetics. *CarA*, *carB*, and *carC* were obtained by standard polymerase chain reactions (PCR) from genomic DNA isolated from *Erwinia carotovora* ssp. *carotovora* (obtained from ATCC 39048).[14] The PCR products were cloned into pT7Blue-3 (Novagen, Madison, WI) and then into pET24a (Novagen) to generate the overexpression vectors pET24a/*carA*, pET24a/*carB*, and pET24a/*carC*. *CarB* was recovered from pET24a/*carB* along with the ribosome binding sequence by *Xba*I-*Hind*III digestion and treatment with T4 DNA polymerase, and this fragment was inserted into the *Not*I site of pET24a/*carA* with the same orientation as *carA* to give the coexpression vector pET24a/*carAB*.

Fermentation of *E. coli* Transformants. *E. coli* BL21 (DE3)pLysS (pET24a/*carAB*) was grown at 37 °C in 50 mL of LB medium on a rotary shaker for 15 h at 300 rpm. The resulting seed culture was used as inoculum (0.2%) for fermentation in the modified *S. marcescens* fermentation medium (L-glutamate 0.5%, NH_4Cl 0.075%, K_2HPO_4 0.2%, $MgSO_4$ 0.05%, NaCl 0.05%, $FeSO_4{\cdot}7H_2O$ 0.0025%, $CaCO_3$ 0.025% and glucose 1%; pH 7.5). Cells were grown in the bioreactor with 3 L of fermentation medium at 37 °C. The simultaneous overexpression of *carA* and *carB* was induced at $OD_{600} = 0.6$ with 1 mM IPTG. The protein overexpression and carbapenam production were carried out at 28 °C for 6 h following the induction.

Isolation of *β*-lactams from *S. marcescens* and *E. coli* fermentations. Extraction and derivatization of the β-lactams produced was accomplished by the methods established by Bycroft.[3]

For the [5,5-^{2}H$_2$,5-^{13}C]-L-proline feeding experiment, ^{13}C NMR analysis was carried out utilizing the crude residue isolated after silica gel chromatography. The resonances for C-5 in the PNB esters of **2**, **3**, and **4** were at δ 53.1, 53.2, and 51.3, respectively.

In the case of the radiolabeled L-proline incorporation experiment, the similarly isolated residue was solubilized in 5 mL of 50/50 acetonitrile/water and further purified by reverse phase HPLC utilizing a Phenomenex Prodigy 10μ C(8)-Semi-Prep column at a flow rate of 5 mL/min (60/40 acetonitrile/water eluant) with 500 μL injections. The eluant was monitored at 320 nm and the PNB esters **2**, **3**, and **4** elute individually between 12 and 16 min. The pure β-lactam containing fractions were collected, frozen, and lyophilized for scintillation counting.

Purification of the β-lactams for PNB derivatized stereochemical correlations was accomplished utilizing normal phase HPLC. The fractions containing β-lactam(s) from the silica gel chromatography

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were evaporated and resolubilized in 5 mL of 30% ethyl acetate/hexane, and 500 μL portions were injected onto a Phenomenex Luna 5μ CN Semi-Prep column with a flow rate of 5 mL/min (30/70 ethyl acetate/hexanes eluant). The eluant was monitored at 320 nm with elution of the β-lactams occurring between 8.5 and 12 min.

Transformation and *carC* Overexpression. pET24a/*carC* was transformed into *E. coli* BL21 (DE3) by electroporation using a Gibco/BRL Cell-Porator Electroporation System (Rockville, MD) according to the manufacturer's instructions. A 50 mL 2xYT seed culture was inoculated with a single colony picked from freshly transformed plates grown at 37 °C for 18 h. Cells were collected by centrifugation and resuspended in 15 mL of 2xYT medium. Then 5 mL of the cell suspension was used to inoculate 3 L of 2xYT medium. Upon $OD_{600} = 0.6$, the temperature was reduced to 20 °C and 1 mM IPTG and 0.4 mM ferrous ammonium sulfate were added. The fermentation was continued for 5 h and the cells were harvested by centrifugation at 4000*g* for 10 min at 4 °C.

Cell-Free Extract of CarC Overexpression. All cell lysis steps were carried out at 0−4 °C. Assays for CarC activity were carried out at 25 °C.

E. coli BL21(DE3) cell paste (4.6 g) harboring overexpressed CarC was suspended in 10 mL of 20 mM sodium phosphate buffer containing 10% glycerol, 2 mM DTT, 1 mM benzamidine, 10 μM EDTA, and 1 mM PMSF, pH 8.0. Lysozyme (10 mg) was then added, and the solution was mixed gently for 5 min. Brij-58 (10 mg solubilized in 1 mL of lysis buffer) was then added and mixing continued for an additional 10 min. DNase/RNase (~1 mg each) was added and the solution gently mixed for 1 h. The resulting suspension was then clarified by centrifugation at 35 000*g* for 30 min at 4 °C.

Assays for CarC activity in the CFE contained the following components: CarC CFE solution, 1 mM ascorbate, 8 mM α-ketoglutarate, 2 mM (3*S*,5*S*)-carbapenam **2**, in a total volume of 1.00 mL incubated at room temperature for 2.5 h. Carbapenem production was detected by visualization of antibiosis on a plate of β-lactam supersensitive *E. coli* strain SC12155, incubated at 28 °C for 24 h. The control without CarC CFE solution contained lysis buffer in its place, and the control for the CFE solution and reaction components contained lysis buffer in place of added **2**.

Carbapenam (2). (2*S*)-Benzyl *N*-(Boc)pyroglutamate (5) was prepared according to a published procedure:[10] 9.42 g, 76%; mp 72−73 °C; $[\alpha]^{25}_D$ −36.4° (*c* 1, $CHCl_3$); (lit.[10] mp 69−70 °C; $[\alpha]^{25}_D$ −37.8° (*c* 1, $CHCl_3$)); TLC (EtOAc/hexanes, 30/70) R_f 0.25. ^{1}H NMR: δ 7.31 (m, 5), 5.16 (abq, J = 12.0 Hz, 2H), 4.61 (dd, J = 9.4 Hz, 3.2 Hz, 1H), 2.55 (m, 1H), 2.44 (m, 1H), 2.29 (m, 1H), 1.97 (m, 1H), 1.37 (s, 9H). ^{13}C NMR: δ 173.1, 171.0, 149.0, 134.9, 128.5, 128.5, 128.4, 83.4, 67.2, 58.8, 30.9, 27.6, 21.3. IR: 2983, 2341, 2256, 1790, 1749, 1716 cm^{-1}. HRMS calcd for $C_{17}H_{21}NO_5$ [M + Na^+]: 342.1312, found 342.1323.

5-Hydroxy-(2*S*)-benzyl *N*-(Boc)prolinate (16) was synthesized according to an established protocol:[11] 23 mL of a 1.0 M solution of Superhydride (23 mmol) in THF was added dropwise to a solution of pyroglutamate **5** (6.11 g, 19.16 mmol) in 200 mL of dry THF at −78 °C under an argon atmosphere and stirred for 30 min. The reaction was quenched by the addition of saturated $NaHCO_3$ (34 mL) and 30% H_2O_2 (4 mL), the temperature was raised to 0 °C, and the solution was stirred for 20 min. The solvent was removed in vacuo, and the aqueous layer was extracted with CH_2Cl_2 (2 × 100 mL). The organic layers were combined, dried over $MgSO_4$, filtered, and concentrated. The residue was purified by silica gel chromatography utilizing 40/60 EtOAc/hexanes as the eluant to afford a colorless oil: 4.54 g, 95% yield; $[\alpha]^{25}_D$ −31.8° (*c* 1, $CHCl_3$); TLC (EtOAc/hexanes, 40/60) R_f 0.30. ^{1}H NMR (mixture of rotamers and diastereomers): δ 7.26 (m, 5H), 5.53 (m, 0.8H), 5.44 (m, 0.3H), 5.10 (m, 2H), 4.33 (t, J = 7.6 Hz, 0.4H), 4.20 (t, J = 8.0 Hz, 0.8H), 3.76 (d, J = 2.8 Hz, 0.8H), 3.28 (d, J = 3.9 Hz, 0.2H), 2.15 (m, 2H), 1.89 (m, 2H), 1.43 (s, 3H), 1.27 (s, 6H). ^{13}C NMR: δ 172.5, 153.6, 135.4, 128.3, 128.3, 128.1, 128.1, 127.9, 127.8, 82.1, 81.9, 80.7, 77.3, 66.7, 66.6, 59.3, 59.0, 33.2, 32.1, 28.1, 27.9, 27.7, 26.9. IR: 3471, 3030, 2981, 1747, 1695, 1382, 1161 cm^{-1}. HRMS calcd for $C_{17}H_{23}NO_5$ [M + NH_4^+]: 339.1920, found 339.1931.

***N*-(Boc)-(2*S*,5*S*)-((*tert*-butyl)carboxymethyl)proline benzyl ester (7)** was synthesized on the basis of an established protocol:[9] *tert*-butyl diethylphosphonoacetate (4.8 mL, 17.15 mmol) was added dropwise to a stirring suspension of 30% KH/mineral oil (2.29 g, 17.15 mmol) in 100 mL of dry DMF. The reaction was stirred at room temperature for 1 h under argon. At this time, carbinolamide **6** (5.5 g, 17.15 mmol) in 100 mL of dry DMF was added to the reaction and allowed to stir overnight. The reaction was quenched by the addition of saturated NH_4-Cl (1 L) and extracted with diethyl ether (2 × 200 mL). The organic layers were collected, dried over $MgSO_4$, filtered, concentrated, and purified by silica gel chromatography with 5% ethyl acetate/hexanes as the eluant to yield the protected carboxymethyl proline as a colorless oil: 6.10 g, 84% yield (7:1 mixture of diastereomers); $[\alpha]^{25}_D$ −46.4° (*c* 1, $CHCl_3$); TLC (EtOAc/hexanes, 20/80), R_f 0.55. ^{1}H NMR (major diastereomer) δ 7.28 (m, 5H), 5.09 (m, 2H), 4.31 (m, 1H), 4.23 (m, 1H), 2.85 (dd, J = 15.4 Hz, 3.6 Hz, 0.68H), 2.66 (dd, J = 14.6 Hz, 2.8 Hz, 0.36H), 2.14 (m, 3H), 1.86 (m, 1H), 1.73 (m, 1H), 1.40 (s, 5H), 1.37 (s, 7H), 1.26 (s, 6H). ^{13}C NMR: δ172.6, 172.3, 170.5, 153.8, 153.3, 135.4, 128.5, 128.3, 128.3, 128.2, 128.1, 128.0, 127.9, 80.4, 80.3, 80.1, 79.9, 66.55, 59.6, 59.3, 54.9, 54.8, 54.7, 40.3, 39.1, 29.5, 28.6, 28.5, 28.1, 28.0, 27.9, 27.1; IR: 2981, 1720, 1694, 1476, 1894, 1368, 1164 cm^{-1}. HRMS calcd for $C_{23}H_{33}NO_6$ [M + H^+]: 420.2386, found 420.2392.

Benzyl-(3*S*,5*S*)-carboxymethyl Prolinate (8). The fully protected carboxymethylproline **7** (5.0 g, 11.9 mmol) was solubilized in 50 mL of TFA and stirred for 30 min at room temperature. The reaction was then concentrated in vacuo, diluted with distilled H_2O, and lyophilized. The residual material was taken up in 0.2 N AcOH, passed through a cation exchange column (Bond Elut, SCX, Varian), and eluted with distilled H_2O. The H_2O fractions were lyophilized to dryness to yield the aminodiacid α-benzyl ester as a white crystalline powder: 3.02 g, 96% yield; mp 67−69 °C (dec); $[\alpha]^{25}_D$ −24.3° (*c* 1, H_2O). ^{1}H NMR (major diastereomer): δ 7.44 (m, 5H), 5.28 (abq, J = 12.0 Hz, 2H), 4.54 (t, J = 8.4 Hz, 1H), 3.95 (m, 1H), 2.64 (dd, J = 10.4 Hz, 2.0 Hz, 2H), 2.48 (m, 1H), 2.23 (m, 1H), 2.12 (m, 1H), 1.76 (m, 1H). ^{13}C NMR: 176.7, 169.2, 169.2, 134.1, 128.4, 127.9, 68.1, 58.6, 58.3, 57.7, 36.7, 36.4, 28.7, 27.8, 27.2, 26.7; IR: 2982, 1744, 1672, 1412, 1201 cm^{-1}. HRMS calcd for $C_{14}H_{17}NO_4$ [M + H^+]: 264.1236, found 264.1240.

(3*S*,5*S*)-Carbapenam Carboxylic Acid Benzyl Ester (9). The amino diacid α-benzyl ester **8** (1.78 g, 6.77 mmol), tris(2,3-dihydro-2-oxobenzoxazol-3-yl)phosphine oxide[12,13] (2.59 g, 6.77 mmol), and TEA (1.85 mL, 15.57 mmol) in 400 mL of dry CH_3CN were heated to reflux and for 6 h. The reaction mixture was concentrated in vacuo, absorbed onto Celite, and purified by repeated silica gel chromatography utilizing 15% ethyl acetate/hexanes as the eluant to give a colorless oil free of any residual diastereomer: 810 mg, 38% yield; $[\alpha]^{25}_D$ −190.8° (*c* 1, $CHCl_3$); TLC (EtOAc/hexanes, 40/60) R_f 0.37. ^{1}H NMR: δ 7.25 (m, 5H), 5.06 (s, 2H), 4.36 (t, J = 7.6 Hz, 1H), 3.75 (m, 1H), 3.15 (dd, J = 15.4 Hz, 5.2 Hz, 1H), 2.53 (dd, J = 15.8 Hz, 1.6 Hz, 1H), 2.58 (m, 1H), 2.26 (m, 2H), 1.53 (m, 1H). ^{13}C NMR: δ 175.9, 170.9, 135.1, 128.3, 128.0, 127.8, 66.6, 58.8, 52.8, 42.3, 35.2, 30.6; IR: 2798, 2252, 1761, 1189 cm^{-1}. HRMS calcd for $C_{14}H_{15}NO_3$ [M^+]: 245.1052, found 245.1054.

(3*S*,5*S*)-Carbapenam Carboxylic Acid (2). The benzyl-protected carbapenam **9** (100 mg, 0.41 mmol) was dissolved in 10 mL of ethyl acetate and degassed with argon for 5 min in a Parr hydrogenation apparatus. Pd/C (20 mg) was added, followed by $NaHCO_3$ (34.3 mg, 0.41 mmol), and hydrogenation was carried out for 90 min at 15 psi. The suspension was then filtered through Celite, and the Celite was washed with 50 mL of CH_2Cl_2, followed by 100 mL of distilled H_2O. The aqueous layer was separated and extracted with another 50 mL of

CH_2Cl_2. The aqueous layer was then collected and lyophilized to leave carbapenam carboxylic acid, sodium salt with the hydrolysis product, (2*S*,5*S*)-carboxymethylproline (**1**), as a contaminant (~10%): 61.4 mg, 85% yield; mp 125 °C (dec); $[\alpha]^{25}_D$ −148.0° (*c* 1, H_2O). ^{1}H NMR: δ 4.17 (t, J = 8.4 Hz, 1H), 3.83 (m, 1H), 3.22 (dd, J = 16.0 Hz, 4.4 Hz, 1H), 2.66 (dd, J = 16.4 Hz, 1.6 Hz, 1H), 2.59 (m, 1H), 2.18 (m, 1H), 2.05 (m, 1H), 1.48 (m, 1H). ^{13}C NMR: 180.1, 179.2, 61.2, 52.9, 40.1, 35.9, 30.1; IR: 3430, 2927, 1742, 1615, 1400 cm^{-1}. ESI-MS calcd for $C_7H_8NO_3$ [M − H$^+$]: 154.1, found 154.2, MS/MS 112 (−ketene).

(3*S*,5*S*)-Carbapenam Carboxylic Acid *p*-Nitrobenzyl Ester (10). Carbapenam, sodium salt **2** (100 mg, 0.56 mmol) was added to 10 mL of dry DMF, followed by the addition of *p*-nitrobenzyl bromide (159 mg, 0.73 mmol). The reaction was stirred at room temperature for 4 h, poured into 100 mL of distilled H_2O, and extracted with EtOAc (2 × 50 mL). The organic extracts were collected, dried over $MgSO_4$, filtered, and concentrated in vacuo. The residue was purified by silica gel chromatography using 15/85 EtOAc/hexanes as eluant to yield a yellow oil which had identical TLC, HPLC, and NMR properties as those assigned to carbapenam isolated and derivatized from *S. marcescens* and the *carAB E. coli* transformant: 130 mg, 80% yield; $[\alpha]^{25}_D$ −168.2° (*c* 1, $CHCl_3$); TLC (EtOAc/hexanes, 40/60) R_f 0.29. ^{1}H NMR: 8.23 (d, J = 8.8 Hz, 2H), 7.52 (d, J = 8.8 Hz, 2H), 5.25 (s, 2H), 4.48 (t, J = 7.6 Hz, 1H), 3.87 (m, 1H), 3.30 (dd, J = 16.0 Hz, 4.8 Hz, 1H), 2.66 (dd, J = 16.0 Hz, 2.0 Hz, 1H), 2.61 (m, 1H), 2.29 (m, 2H), 1.56 (m, 1H). ^{13}C NMR: 176.1, 170.8, 147.4, 142.4, 128.1, 123.6, 65.1, 58.7, 52.9, 42.3, 35.1, 30.9; IR: 3689, 2957, 2360, 2340, 2254, 1761, 1607, 1525, 1348 cm^{-1}. HRMS calcd for $C_{14}H_{14}N_2O_5$ [M$^+$]: 290.0903, found 290.0896.

L-Proline. *N*-Cbz-L-Glutamic acid 1-methyl ester was prepared according to a published procedure:[19,20] 85% yield; $[\alpha]^{25}_D$ −24.5° (*c* 1, CH_3Cl); TLC (EtOAc/hexanes/AcOH, 40/60/1) R_f 0.32. ^{1}H NMR: δ 7.34 (m, 5H), 5.45 (d, J = 8.0 Hz, 1H), 4.40 (dt, J = 13.2, 5.2 Hz, 1H), 3.75 (s, 3H), 2.41 (m, 2H), 2.21 (m, 1H), 1.98 (m, 1H). ^{13}C NMR: δ 177.9, 172.3, 156.0, 136.0, 128.5, 128.2, 128.1, 67.1, 53.1, 52.6, 29.8, 27.4. IR: 3035, 2956, 1718, 1509, 1438, 1347, 1214 cm^{-1}. HRMS calcd for $C_{14}H_{17}NO_6$ [M + H$^+$]: 296.1135, found 296.1141.

4-Bromo-(2*S*)-Cbz-aminomethylbutanoate (11) was prepared according to a published procedure:[22] 58% yield; mp 61 °C; $[\alpha]^{25}_D$ −40.3° (*c* 1, DMF); (lit.[22] mp 63−64 °C, $[\alpha]^{25}_D$ −40.8° (*c* 1, DMF)); TLC (EtOAc/hexanes, 20/80) R_f 0.34. ^{1}H NMR: δ 7.35 (m, 5H), 5.39 (d, J = 7.3 Hz, 1H), 5.11 (s, 2H), 4.51 (dt, J = 12.8, 4.8 Hz, 1H), 3.76 (s, 3H), 3.42 (t, J = 6.8 Hz, 2H), 2.44 (m, 1H), 2.25 (m, 1H). ^{13}C NMR: δ 172.9, 156.9, 137.0, 129.7, 129.4, 129.2, 68.3, 53.9, 53.8, 36.8, 29.1; IR 2252, 1723, 1507 cm^{-1}. HRMS calcd for $C_{13}H_{16}BrNO_4$ [M$^+$]: 329.0263, found 329.0271.

4-Cyano-(2*S*)-Cbz-aminomethylbutanoate (12). Sodium cyanide (2.13 g, 4.27 mmol, 1.2 equiv) and 8.9 mL of DMSO were warmed for 20 min at 60 °C with stirring. The bromide **11** (11.74 g, 35.56 mmol, 1 equiv) was added so that the reaction temperature did not exceed 60 °C. After the addition was complete, the mixture was stirred at 60 °C for 3 h. The reaction mixture was diluted with 30 mL of distilled H_2O and extracted with ethyl ether (2 × 150 mL). The organic extract was washed with 1 N HCl (1 × 50 mL) and brine (1 × 25 mL), dried over magnesium sulfate, filtered, and evaporated. The product was absorbed onto Celite and purified by silica gel chromatography using 20/80 ethyl acetate/hexane as the eluant: 8.73 g, 88% yield; mp 42 °C; $[\alpha]^{25}_D$ −21.3° (*c* 0.6, MeOH); (lit.[31] mp 42−43 °C; $[\alpha]^{25}_D$ −21° (*c* 0.6, MeOH)); TLC (EtOAc/hexanes), 20/80 R_f 0.25. ^{1}H NMR: δ 7.35 (m, 5H), 5.56 (d, J = 7.1 Hz, 1H), 5.11 (s, 2H), 4.44 (dt, J = 12.3 Hz, J = 4.8 Hz, 1H), 3.77 (s, 3H), 2.42 (m, 2H), 2.29 (m, 1H), 2.02 (m, 2H). ^{13}C NMR: δ 171.2, 155.8, 135.8, 128.5, 128.3, 128.1, 118.6, 67.3, 52.9, 52.7, 28.5, 13.5. ^{13}C NMR (with ^{13}C label at C5): δ 171.2, 155.8, 135.8, 128.5, 128.3, 128.1, (C5) 118.6, 67.2, 52.9, 52.7, 28.5, 13.5 (d, J = 57.2 Hz). IR 2359, 2253, 1723, 1506 cm^{-1}. HRMS calcd for $C_{14}H_{16}N_2O_4$ [M$^+$]: 276.1110, found 276.1115.

(31) Van, T. T.; Korjo, E.; Grzonka, Z. *Tetrahedron* **1977**, *33*, 2299−2302.

L-Glutamic acid (13) was prepared by a modification of a previously published procedure.[23] 4-Cyano-(2*R*)-Cbz-aminomethylbutanoate (12.5 g, 45.1 mmol) was added to 150 mL of acetic acid and 75 mL of concentrated HCl and heated to reflux for 6 h. This solution was concentrated in vacuo to afford a crude salt. Water was added to the residue and the solution was again evaporated; this was repeated two more times to remove any remaining volatile acids. The residue was then dissolved in 6 mL of 2% HCl and the suspension was adjusted to pH = 5 with pyridine, diluted with 160 mL of ethanol, and cooled to −20 °C overnight. The precipitate was filtered and the mother liquor brought back to −20 °C and filtered again: 4.22 g, 63% yield; mp 198 °C (dec); $[\alpha]^{25}_D$ +30.6° (*c* 1, 6 N HCl); (lit.[32] mp 200 °C (dec); $[\alpha]_D^{22.4}$ +31.4° (6 N HCl)). ^{1}H NMR: δ 3.76 (t, J = 6.4 Hz, 1H), 2.50 (m, 2H), 2.11 (m, 2H). ^{13}C NMR: δ 182.9, 181.6, 57.0, 34.3, 29.8. HRMS calcd for $C_5H_{10}NO_4$ [M + H$^+$]: 148.0604, found 148.0600. (5-^{13}C) $[\alpha]^{25}_D$ +30.8° (*c* 1, 6 N HCl). ^{1}H NMR: δ 3.81 (t, J = 6.4 Hz), 2.55 (m, 2H), 2.14 (m, 2H). ^{13}C NMR: δ (C5) 182.8, 55.9, 34.2 (d, J = 51.9 Hz), 31.7. [M + H$^+$]: 149.

(*S*)-*N*-Benzylglutamic acid was prepared according to a published procedure:[24] 65% yield; mp 160−162 °C; $[\alpha]^{25}_D$ +18.3° (*c* 1, 6 N HCl); (lit.[24] mp 162−163 °C; $[\alpha]^{25}_D$ +18.5° (*c* 1, 6 N HCl)). ^{1}H NMR: δ 7.43 (m, 5H), 3.99 (d, J = 12.8 Hz, 1H), 3.91 (d, J = 12.8 Hz, 1H), 3.36 (t, J = Hz, 6.0 1H), 2.24 (m, 2H), 1.95 (m, 2H). ^{13}C NMR: δ 181.9, 177.3, 134.5, 129.5, 129.1, 128.9, 62.4, 50.8, 34.1, 27.8. HRMS calcd for $C_{12}H_{16}NO_4$ [M + H$^+$]: 238.1074, found 238.1076.

(*S*)-*N*-Benzylpyroglutamic acid was prepared according to a published procedure:[24] 84% yield; mp 71−75 °C; $[\alpha]^{25}_D$ +54.3° (*c* 2.32, MeOH); (lit.[24] mp 92−93 °C; $[\alpha]^{25}_D$ +54.6° (*c* 2.32, MeOH)). ^{1}H NMR: δ 11.7 (br. s, 1H), 7.26 (m, 5H), 5.24 (d, J = 14.4 Hz, 1H), 4.0 (dd, J = 9.6 Hz, J = 3.2 Hz, 1H), 3.97 (d, J = 14.4 Hz, 1H), 2.63 (m, 1H), 2.51 (m, 1H), 2.27 (m, 1H), 2.17 (m, 1H). ^{13}C NMR: δ 176.7, 173.8, 135.1, 128.7, 128.4, 127.8, 58.7, 45.6, 29.5, 22.7. HRMS calcd for $C_{12}H_{13}NO_3$ [M$^+$]: 219.0895, found 219.0900.

(*S*)-*tert*-butyl *N*-benzylpyroglutamate was prepared according to a published procedure:[24] 62% yield; mp 73−74 °C; $[\alpha]^{25}_D$ +16.1° (*c* 2.6, CH_3Cl); (lit.[24] mp 62−63 °C; $[\alpha]^{25}_D$ +16.2° (*c* 2.6, CH_3Cl)); TLC (EtOAc/hexanes, 25/75) R_f 0.17. ^{1}H NMR: δ 7.25 (m, 5H), 4.97 (d, J = 15.2 Hz, 1H), 3.88 (d, J = 15.2 Hz, 1H), 3.76 (dd, J = 9.2 Hz, 3.2 Hz, 1H), 2.52−1.96 (m, 4H), 1.37 (s, 9H). ^{13}C NMR: δ 176.1, 171.9, 137.0, 129.7, 129.5, 128.7, 83.2, 60.5, 46.5, 30.6, 28.9, 23.9. IR 2249, 1733, 1684, 1417 cm^{-1}. HRMS calcd for $C_{16}H_{21}NO_3$ [M$^+$]: 275.1521, found 275.1525.

(*S*)-*N*-Benzyl-5-thioxoproline *tert*-butyl ester (14) was prepared according to a published procedure:[24] 82% yield; mp 75−78 °C; $[\alpha]^{25}_D$ +195° (*c* 1.85, CH_3Cl); (lit.[24] mp 78−79 °C; $[\alpha]^{25}_D$ +190° (*c* 1.85, CH_3Cl)); TLC (EtOAc/hexanes, 25/75) R_f 0.48. ^{1}H NMR: δ 7.28 (m, 5H), 5.76 (d, J = 14.4 Hz, 1H), 4.25 (d, J = 14.4 Hz, 1H), 4.11 (ddd, J = 9.19 Hz, J = 3.20 Hz, J = 1.99 Hz, 1H), 3.06 (m, 2H), 2.17 (m, 1H), 2.06 (m, 1H), 1.40 (s, 9H). ^{13}C NMR: δ 203.5, 169.1, 134.5, 128.7, 128.5, 128.0, 82.7, 66.2, 50.3, 43.3, 27.8, 24.7. IR: 2983, 2227, 1734, 1474 cm^{-1}. HRMS calcd for $C_{16}H_{21}NO_2S$; [M$^+$] 291.1293, found 291.1298.

(*S*)-*N*-Benzylproline *tert*-Butyl Ester (15). The thiolactam **14** (2.75 g, 9.43 mmol) was dissolved in 23 mL of dry CH_3CN, treated with methyl iodide (2.34 mL, 37.5 mmol), and stirred until the thiolactam was consumed as monitored by TLC (~18 h). The solvent was then evaporated and the residue was dried under high vacuum for 1 h. The resulting thioiminium salt was then dissolved in 30 mL of methanol and immediately treated with sodium borohydride (173 mg, 3.75 mmol) and stirred at room temperature for 3 h. The reaction mixture was added to 42 mL of 20% NaOH and extracted with ether (2 × 20 mL). The ether extracts were washed with 1 N potassium bisulfate (2 × 15 mL). The combined aqueous layers were adjusted to pH = 12 with 20%

(32) *The Merck Index*, 12th ed.; Merck Research Laboratories: Whitehouse Station, NJ, 1996.

NaOH and re-extracted with ether (3 × 15 mL). The ether extracts were dried over anhydrous potassium carbonate, filtered, concentrated, and purified by silica gel chromatography using 15% EtOAc/hexanes as the eluant to yield a colorless oil: 1.65 g, 67% yield; $[\alpha]^{25}_D$ −68.33° (*c* 4, $CHCl_3$), (lit.[33] for methyl ester, $[\alpha]^{25}_D$ −85.7° (*c* 4, $CHCl_3$)); TLC (EtOAc/hexanes, 15/85) R_f 0.22. ^{1}H NMR: δ 7.27 (m, 5H), 3.96 (d, J = 12.8 Hz, 1H), 3.48 (d, J = 12.8 Hz, 1H), 3.11 (dd, J = 8.8 Hz, 6.0 Hz, 1H), 2.97 (abq, J = 8.4 Hz, 1H), 2.33 (m, 1H), 2.05 (m, 1H), 1.90 (m, 2H), 1.73 (m, 1H), 1.45 (s, 9H). ^{13}C NMR: δ 173.1, 138.7, 128.9, 127.9, 126.8, 80.3, 65.6, 58.3, 52.9, 28.9, 27.9, 22.8. HRMS calcd for $C_{16}H_{23}NO_2$, [M^+] 261.1729, found 261.1731.

(*S*)-*N*-Benzylproline. *N*-Benzylproline *tert*-butyl ester **15** (1.65 g, 6.3 mmol) was dissolved in 10 mL of trifluoroacetic acid and stirred at room temperature for 30 min. The TFA was evaporated and the residue dried under vacuum for 1 h. The residue was then taken up in distilled H_2O and lyophilized. The crystalline residue was triturated with diethyl ether to yield a fine white powder: 971 mg, 75%; mp 167 °C; $[\alpha]^{25}_D$ −29.3° (*c* 1, EtOH); (lit.[33] mp 163.7−165.7 °C; $[\alpha]^{25}_D$ −26.9° (*c* 1, EtOH)). ^{1}H NMR: δ 7.46 (m, 5H), 4.32 (dd, J = 30.0 Hz, 12.8 Hz, 2H), 3.95 (dd, J = 9.6 Hz, 6.8 Hz, 1H), 3.60 (m, 1H), 3.23 (m, 1H), 2.45 (m, 1H), 1.99 (m, 3H). ^{13}C NMR: δ 173.6, 130.6, 130.1, 130.1, 129.3, 68.3, 58.4, 54.7, 28.9, 22.9. HRMS calcd for $C_{12}H_{15}NO_2$ [M^+]: 205.1103, found 205.1107.

(33) Rispens, M. T.; Gelling, O. J.; de Vries, A. H. M.; Meetama, A.; van Bolhuis, F.; Feringa, B. L. *Tetrahedron* **1996**, *52*, 3521−3546.

L-Proline (16). *N*-Benzyl-L-proline (971 mg, 4.7 mmol) was dissolved in 7 mL of ethanol and sparged with argon for 15 min in a Parr hydrogenation flask. Pearlman's catalyst (146 mg, 15% loading) was added ,and the mixture was hydrogenated at 60 psi for 2 d. The reduction mixture was filtered through Celite and washed with several portions of ethanol. The ethanol fractions were evaporated, and the resulting residue was dissolved in distilled H_2O and lyophilized. The resulting crystalline residue was triturated in diethyl ether to yield a fine white powder: 463 mg, 85% yield, mp 228 °C; $[\alpha]^{25}_D$ −82.3° (*c* 0.57, 0.5 N HCl); (lit.[32] mp 220−222 °C; $[\alpha]_D^{23.4}$ −85.0° (*c* 0.57, 0.5 N HCl)). ^{1}H NMR: δ 4.08 (m, 1H), 3.29 (m, 2H), 2.29 (m, 1H), 2.05 (m, 3H). ^{13}C NMR: δ 174.7, 61.3, 46.1, 29.1, 23.8. HRMS calcd for $C_5H_9NO_2$ [$M + H^+$]: 116.0706, found 116.0705.

[5,5-$^2H_2$,5-^{13}C]: $[\alpha]^{25}_D$ −84.1° (*c* 0.57, 0.5 N HCl). ^{1}H NMR: δ 4.12 (m, 1H), 3.52 (m, 0.14H), 2.34 (m, 1H), 2.0 (m, 3H). ^{13}C NMR: δ 176.6, 61.3, 45.9 (5,5-d_1, t, J = 22.9 Hz), 45.6 (5,5-d_2, p, J = 22.4 Hz), 29.0, 23.6 (d, J = 33.6 Hz). [$M + H^+$]: 119.

Acknowledgment. We are grateful to the National Institutes of Health for financial support (AI14937), to Dr. Brian O. Bachmann for his advice early in this project, and to Dr. Barbara Gerratana for frequent discussions and comments on the manuscript.

JA034248A