JOC*Article*

Biosynthesis of 4-Methylproline in Cyanobacteria: Cloning of *nosE* and *nosF* Genes and Biochemical Characterization of the Encoded Dehydrogenase and Reductase Activities

Hendrik Luesch, Dietmar Hoffmann, Joan M. Hevel, Julia E. Becker, Trimurtulu Golakoti, and Richard E. Moore*

Department of Chemistry, University of Hawaii at Manoa, Honolulu, Hawaii 96822

moore@gold.chem.hawaii.edu

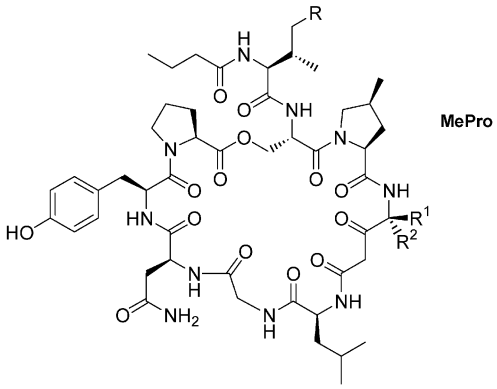
Received September 25, 2002

The biosynthesis of the unusual amino acid 4-methylproline in the *Nostoc* genus of cyanobacteria was investigated on the genetic and enzymatic level. Two genes involved in the biosynthesis were cloned and the corresponding enzymes, a zinc-dependent long-chain dehydrogenase and a Δ^1-pyrroline-5-carboxylic acid (P5C) reductase homologue, were overexpressed in *Escherichia coli* and biochemically characterized. Putative substrates were synthesized to test enzyme substrate specificities, and deuterium labeling studies were carried out to reveal the stereospecificities of the enzymatic reactions with respect to the substrates as well as to the coenzymes.

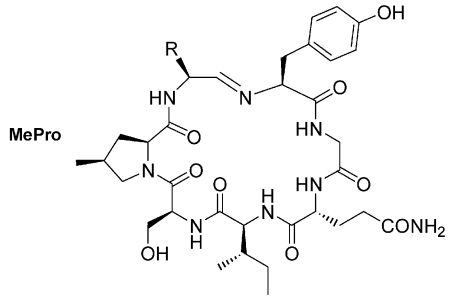
Introduction

Cyanobacteria are prolific producers of secondary metabolites of peptidic nature that are commonly modified by the incorporation of polyketide portions and then considered peptide-polyketide hybrids.[1] Their small size, modification, cyclic nature, *N*-methylation, and the presence of hydroxy acids, unusual amino acids, D-amino acids, or unusual residues indicate that those metabolites are presumably produced nonribosomally via large modular enzyme complexes, called nonribosomal peptide synthetases (NRPSs)[2] and type I polyketide synthases (PKSs).[3] For most biosynthetic gene clusters, the organization and order of the modules on the chromosome or plasmid maps in a 1:1 manner to the sequence of the product (collinearity rule).[2,3] The clustering of biosynthetic genes and the involvement of PKS (type I) and NRPSs in the biosynthesis of cyanobacterial metabolites has recently been demonstrated for microcystin.[4]

We have isolated the NRPS/PKS hybrid gene cluster coding for the biosynthesis of nostopeptolides A[5] (GenBank accession no. AF204805) in the terrestrial cyanobacterium *Nostoc* sp. GSV224.[6] The biosynthetic gene cluster appears to follow the collinearity rule as evidenced by either predicted (substrate-binding pocket model)[7] or experimentally determined (substrate specificity of over-



nostopeptolide A1	$R = CH_3$	$R^1 = CH_2CH(CH_3)_2$; $R^2 = H$
nostopeptolide A2	$R = H$	$R^1 = CH_2CH(CH_3)_2$; $R^2 = H$
nostopeptolide A3	$R = CH_3$	$R^1 = H$; $R^2 = CH_2CH(CH_3)_2$

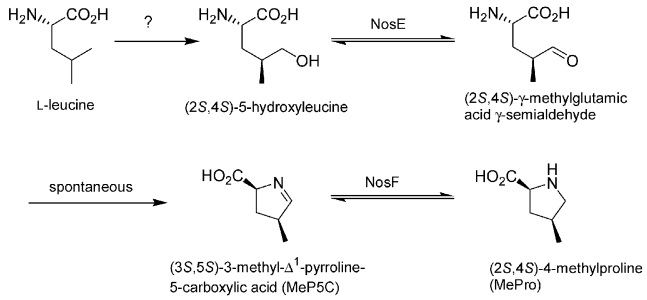


nostocyclopeptide A1 R = *i*-Pr
nostocyclopeptide A2 R = Bn

(1) For a recent review, see: Gerwick, W. H.; Tan, L. T.; Sitachitta, N. In *The Alkaloids*; Cordell, G. A., Ed.; Academic Press: San Diego, CA, 2001; Vol. 57, pp 75−184.
(2) For reviews, see: (a) Marahiel, M. A.; Stachelhaus, T.; Mootz, H. D. *Chem. Rev.* **1997**, *97*, 2651−2673. (b) von Döhren, H.; Keller, U.; Vater, J.; Zocher, R. *Chem. Rev.* **1997**, *97*, 2675−2705.
(3) For review, see: Khosla, C. *Chem. Rev.* **1997**, *97*, 2577−2590.
(4) Tillett, D.; Dittmann, E.; Erhard, M.; von Döhren, H.; Börner, T.; Neilan, B. A. *Chem. Biol.* **2000**, *7*, 753−764.
(5) Golakoti, T.; Yoshida, W. Y.; Chaganty, S.; Moore, R. E. *Tetrahedron* **2000**, *56*, 9093−9102.
(6) Hoffmann, D.; Hevel, J. M.; Moore, R. E. Manuscript in preparation.

10.1021/jo026479q CCC: $25.00
Published on Web 11/28/2002

SCHEME 1. Presumed Biosynthesis of the (2*S*,4*S*)-4-Methylproline Moiety in the Nostopeptolides A



expressed NRPSs measured by an ATP-[^{32}P]PPi exchange enzyme assay)[6] data. The unusual amino acid (2*S*,4*S*)-4-methylproline (MePro) is incorporated into the chemical structures of the nostopeptolides. Some of the genes responsible for the biosynthesis of this unusual amino acid appeared to be embedded in the 3′ end of the nostopeptolide gene cluster. The *nosE* gene showed strong homology to zinc-dependent long-chain alcohol dehydrogenases and the *nosF* gene to Δ^1-pyrroline-5-carboxylic acid (P5C) reductases.[6] This suggested that MePro biosynthesis proceeded via a P5C homologue (Scheme 1) in analogous fashion to 3-methylproline biosynthesis. Feeding experiments have demonstrated that 3-methylproline originates from isoleucine in the cyanobacterial metabolite scytonemin A[8,9] and in fungal metabolites of the paraherquamide family.[10] This suggested that MePro probably arises ultimately from leucine (Scheme 1). A feeding experiment with GSV224 using [1-^{13}C]L-leucine supported this hypothesis since label was incorporated into C-1 of the MePro unit in nostopeptolide A1. Furthermore, the enrichment was comparable with incorporation into C-1 of the leucine unit and C-3 of the modified leucine unit (1%). However, the only uncharacterized open-reading frame in the nostopeptolides A gene cluster, ORF1, showed no homology to any type of oxidizing enzyme. Enzymes for the biosynthesis of the presumed NosE substrate, (2*S*,4*S*)-5-hydroxyleucine, could also be encoded by (a) gene(s) outside the cluster, and ORF1 might have a different, e.g., regulatory function. The primary NosE reaction product would presumably cyclize spontaneously as described for glutamic acid γ-semialdehyde in proline biosynthesis.[11] Finally, MePro synthesis would be completed by the NosF-catalyzed reduction of the pyrroline ring.

In this paper we report the overexpression and kinetic characterization of NosE and NosF, two proteins involved in the biosynthesis of MePro. The focus is on the determination of the substrate specificity of these enzymes and of the stereospecificity of the enzymatic reactions. More recently we have also isolated and sequenced the gene cluster encoding the biosynthesis of another MePro-containing cyanobacterial metabolite, nostocyclopeptide A[12] from Nostoc sp. ATTC53789 (GenBank accession no. AY167420).[13] Genes homologous to *nosE* (*ncpD*, 97% identical and 98% positive on protein level) and *nosF* (*ncpE*, 88% identical and 92% positive on protein level) were identified, and the enzyme activity of NcpD is also presented in this paper.

Results and Discussion

Overexpression of NosE and NosF. Proteins encoded by the *nosE* and *nosF* genes were overexpressed in *E. coli* as *N*-His$_6$-tagged fusion proteins and purified on Ni-NTA resin (Figure 1). NosE and NosF were easily purified to >85% purity, were stable, and migrated as distinct bands of ~35 (NosE) and ~30 kDa (NosF).

Characterization of NosE. Scheme 2 outlines the synthetic strategy applied to efficiently produce the putative natural substrate of NosE, (2*S*,4*S*)-5-hydroxyleucine (**17**) (based on the MePro stereochemistry in the nostopeptolides), and its stereoisomers **18**−**20** in enantiomerically pure form. The reaction sequence is derived from a synthesis reported for 4-methylprolines.[14] The corresponding nonmethylated compounds, (2*S*)- and (2*R*)-5-hydroxy-2-aminovaleric acid (**15** and **16**, Hava), were to be evaluated as well for their potential to serve as NosE substrates. For Hava **15** and **16**, the alkylation step was simply omitted (Scheme 2).

Compounds **17**−**20** were tested as NosE substrates in activity assays that followed NADH formation. The 2*S*,4*S* isomer **17** was the only stereoisomer accepted by NosE. Due to the anticipated instability of the product (3*S*,5*S*)-3-methyl-P5C (MeP5C), the assay product was not isolated but was directly converted to (2*S*,4*S*)-MePro by addition of NosF (coupled assay). The product of the coupled enzyme assay was isolated and identified as (2*S*,4*S*)-MePro by NMR and chiral HPLC analysis. The 2*S* isomer of Hava (**15**) was accepted as a substrate, demonstrating a similar k_{cat}, but a $K_{m,app}$ that was about 5 times greater than that for the methylated natural substrate **17**. The enzymatic oxidation was again proven by a coupled assay as above and subsequent isolation of L-proline. The 2*R* isomer of Hava (**16**) was not a substrate. Michaelis−Menten parameters are provided in Table 1. NosE activity was dependent on Zn^{2+} as a cofactor, which is consistent with the characteristic small subunit size and the homology of NosE to other zinc-dependent (group I, long-chain) dehydrogenases.[15] NosE activity employed NAD^+ and not $NADP^+$ as a coenzyme and exhibited the highest activity at pH 10 and 42 °C.

In summary, the dehydrogenase activity of NosE was confirmed. The (2*S*,4*S*) stereochemistry of the substrate is an absolute requirement for NosE while the lack of the methyl group at C-4 is tolerated. The same conclusion was drawn from data obtained for the NosE homologue,

(7) (a) Stachelhaus, T.; Mootz, H. D.; Marahiel, M. A. *Chem. Biol.* **1999**, *6*, 493−505. (b) Challis, G. L.; Ravel, J.; Townsend, C. A. *Chem. Biol.* **2000**, *7*, 211−224.

(8) Helms, G. L.; Moore, R. E.; Niemczura, W. P.; Patterson, G. M. L. *J. Org. Chem.* **1988**, *53*, 1298−1307.

(9) Hemscheidt, T. K. (University of Hawaii at Manoa), personal communication.

(10) Stocking, E. M.; Martinez, R. A.; Silks, L. A.; Sanz-Cervera, J. F.; Williams, R. M. *J. Am. Chem. Soc.* **2001**, *123*, 3391−3392.

(11) Kramer, J. J.; Henslee, J. G.; Wakabayashi, Y.; Jones, M. E. *Methods Enzymol.* **1985**, *113*, 113−120.

(12) Golakoti, T.; Yoshida, W. Y.; Chaganty, S.; Moore, R. E. *J. Nat. Prod.* **2001**, *64*, 54−59.

(13) Becker, J. E.; Moore, R. E. Manuscript in preparation.

(14) Koskinen, A. M. P.; Rapoport, H. *J. Org. Chem.* **1989**, *54*, 1859−1866.

(15) For review, see: Reid, M. F.; Fewson, C. A. *Crit. Rev. Microbiol.* **1994**, *20*, 13−56.

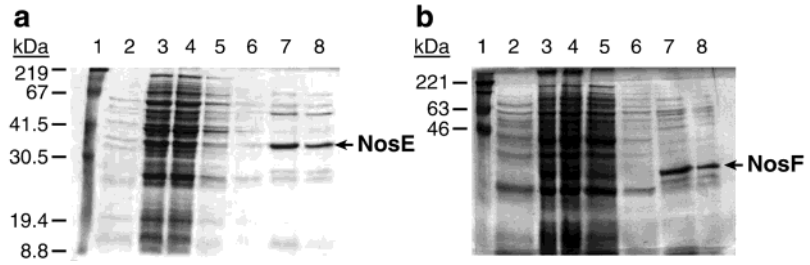
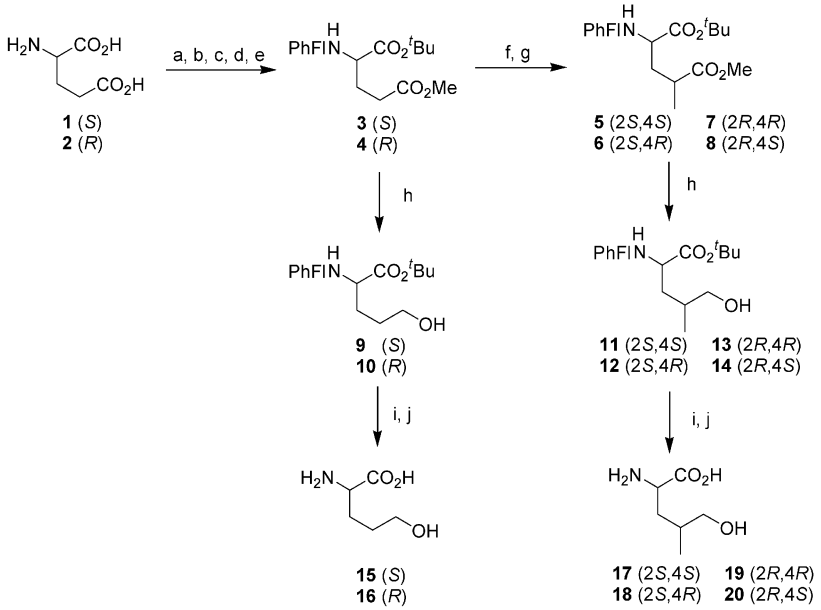


FIGURE 1. Heterologous expression and purification of NosE and NosF: (a) *N*-His_6-tagged NosE and (b) *N*-His_6-tagged NosF overproduced in *E. coli* BL21(DE3)pLysS. Lane 1, MW standard; lane 2, soluble cell extract without IPTG induction; lane 3, soluble proteins after IPTG induction; lane 4, Ni-NTA column flow-through; lane 5, first wash; lane 6, second wash; lanes 7 and 8, purified proteins (eluates 1 and 2).

SCHEME 2. Synthesis of Putative NosE Substrates[a]



[a] Conditions: (a) AcOCl, MeOH; (b) pyridine; (c) TMSCl; (d) Et_3N, $Pb(NO_3)_2$, PhFlBr; (e) *O-tert*-butyl *N*,*N*′-diisopropylisourea; (f) KHMDS, −78 °C; (g) MeI; (h) $LiAlH_4$, −78 °C; (i) TFA; (j) Dowex-50W (H^+), 4 N NH_4OH.

TABLE 1. Kinetic Data for the Enzymatic Oxidation of NosE and NcpD Substrates[a]

	NosE		NcpD	
substrate	$K_{m,app}$ (M)	$k_{cat}/K_{m,app}$ (M^{-1} s^{-1})	$K_{m,app}$ (M)	$k_{cat}/K_{m,app}$ (M^{-1} s^{-1})
(2*S*,4*S*)-5-OH-Leu (**17**)	$(2.4 \pm 0.16) \times 10^{-4}$	$(2.5 \pm 0.45) \times 10^{3}$	$(6.6 \pm 1.1) \times 10^{-4}$	$(7.6 \pm 1.3) \times 10^{2}$
(*S*)-Hava (**15**)	$(1.2 \pm 0.078) \times 10^{-3}$	$(5.0 \pm 0.90) \times 10^{2}$	$(1.3 \pm 0.42) \times 10^{-3}$	$(1.3 \pm 0.40) \times 10^{2}$

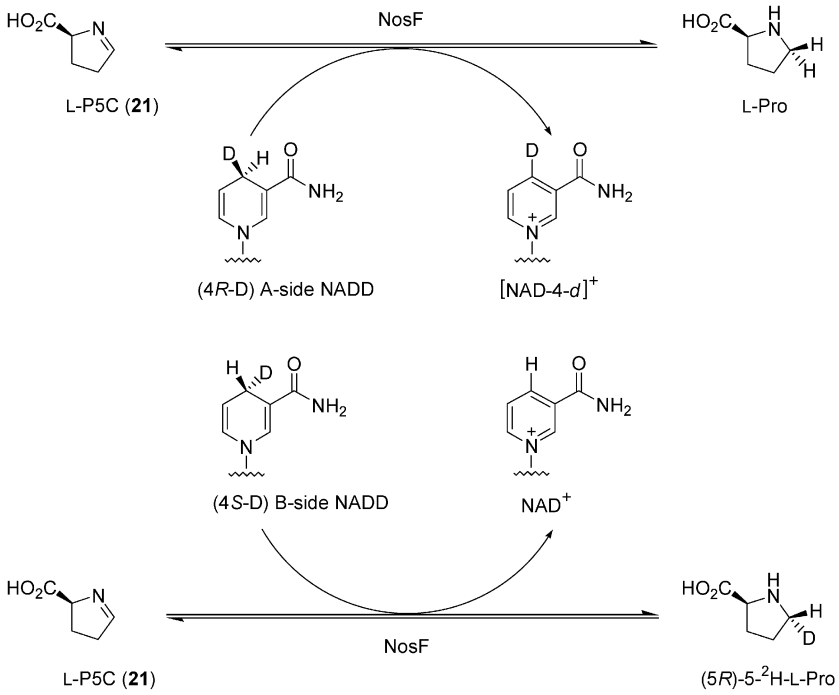
[a] At 2 mM NAD^+, 42 °C, 1 mM $ZnSO_4$, in 100 mM glycine buffer (pH 10).

NcpD, catalyzing the oxidation step in the biosynthesis of MePro in nostocyclopeptide A (see Table 1 for kinetic data).

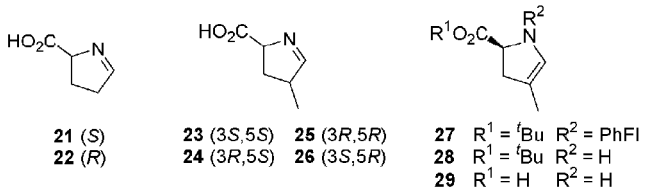
Characterization of NosF. Due to the high homology of NosF to P5C reductases and the ease of generating P5C ($NaIO_4$ oxidation of δ-hydroxylysine),[16] P5C was evaluated as a potential substrate for NosF. Addition of DL-P5C (**21**/**22**) to enzyme reactions resulted in the oxidation of NADH or NADPH in a concentration-dependent fashion, although NADH was the preferred nicotinamide. NosF activity displayed Michaelis−Menten behavior ($K_{m,app}$ = 150 μM, $k_{cat}/K_{m,app} = 3.5 \times 10^4$ M^{-1} s^{-1}, pH 8). At this point it was still unclear if both enantiomers of P5C (**21** and **22**) were substrates of NosF. Consequently, the proline generated in enzymatic reactions was isolated and shown to be exclusively of L stereochemistry by chiral HPLC analysis. Therefore it was concluded that L-P5C (**21**) is a substrate for NosF, while D-P5C (**22**) is not. Since the ratio of **21** and **22** was 1:1 and assuming the D-isomer is not an inhibitor, then one can estimate the $K_{m,app}$ of the pure L-P5C (**21**) at roughly 75 μM. The deduced specificity of NosF was confirmed by employing the enantiomerically pure compounds in NosF assays (data not shown). Herefore, L-P5C (**21**) and D-P5C (**22**) were synthesized by chromic acid

(16) (a) Williams, I.; Frank, L. *Anal. Biochem.* **1975**, *64*, 85−97. (b) Wu, G. Y.; Seifter, S. *Anal. Biochem.* **1975**, *67*, 413−421.

SCHEME 3. Determination of the Stereospecificity of the NADH Reduction by NosF Using Stereospecifically Deuterated NADH



oxidation of Hava **15** and **16**, respectively, similarly as described for α-aminoadipic-δ-semialdehyde.[17]



Chromic acid oxidation of 5-hydroxyleucines (**17**−**20**) provided the corresponding methylated compounds (**23**−**26**, MeP5C). Although preparations of both the natural substrate (**23**) and the diastereomer **24** supported NosF activity, the activity observed with the natural substrate was approximately three times faster at any given concentration of substrate (data not shown). Since (2*S*,4*S*)-MePro was the only product formed in both NosF reactions, i.e., starting from **23** and **24** (determined by NMR and chiral HPLC of the isolated assay product), it appears that the apparent activity of (3*R*,5*S*)-MeP5C (**24**) is due to partial epimerization at C-3 (∼30%) under the strong acidic conditions of the substrate synthesis (4 N HCl, 80 °C, 1 h). Preliminary data suggest that compound **24** may be functioning as an inhibitor of NosF activity, such that more studies will be required to obtain kinetic parameters.

PDC oxidation of compound **11** or of the corresponding selectively *N*-deprotected compound led to formation of the enamine tautomers of P5C derivatives, **27** and **28**, respectively.[18] Following TFA-mediated deprotection, the resulting enamine **29** was tested as a substrate in NosF assays; however, no catalysis was observed. This also indicated that there is no significant equilibrium between both tautomers under assay conditions.

In summary, the predicted reductase activity of NosF was demonstrated. The L-configuration (5*S*) of the substrate and the *S* stereochemistry at C-3 of MeP5C are essential for activity. On the other hand, the absence of the C-3 methyl group is tolerated by NosF.

Stereospecificity of NADH Reduction by NosF. For reasons that become obvious below, the stereospecificity of NosF was investigated prior to the stereospecificity of NosE. To determine whether NosF was A-side or B-side specific, (4*R*-^{2}H)- and (4*S*-^{2}H)-NADH (A-side and B-side NADD) were synthesized. The stereospecificities of horse liver dehydrogenase and glucose dehydrogenase were exploited to generate A-side and B-side NADD, respectively, using established procedures.[19]

Since the stereospecificity of an enzyme is independent of its substrate,[20] stereospecificity studies were carried out with the nonmethylated substrate, L-P5C (**21**), employed as a racemic mixture with **22**. When NosF was incubated with DL-P5C (**21**/**22**) and A-side NADD, the label was retained in the oxidized coenzyme (see Scheme 3). Inspection of the ^{1}H NMR spectrum of the crude product led to this conclusion. The aromatic region lacked the signal for H-4 of the pyridine moiety of NAD$^+$; thus NAD$^+$ contained deuterium at this position.[21] This result was confirmed after isolation of L-proline, which was deuterium-free (see Scheme 3 and Figure 2a), indicating that a hydride was transferred during the reaction.

(17) Jones, E. E.; Broquist, H. P. *J. Biol. Chem.* **1965**, *240*, 2531−2536.
(18) Luesch, H. Ph.D. Thesis, University of Hawaii, 2002.
(19) Viola, R. E.; Cook, P. F.; Cleland, W. W. *Anal. Biochem.* **1979**, *96*, 334−340.
(20) You, K.; Arnold, L. J., Jr.; Allison, W. S.; Kaplan, N. O. *Trends Biochem. Sci.* **1978**, *3*, 265−268.
(21) For a similar NMR analysis and spectra, see: Ottolina, G.; Riva, S.; Carrea, G.; Danieli, B.; Buckmann, A. F. *Biochim. Biophys. Acta* **1989**, *998*, 173−178.

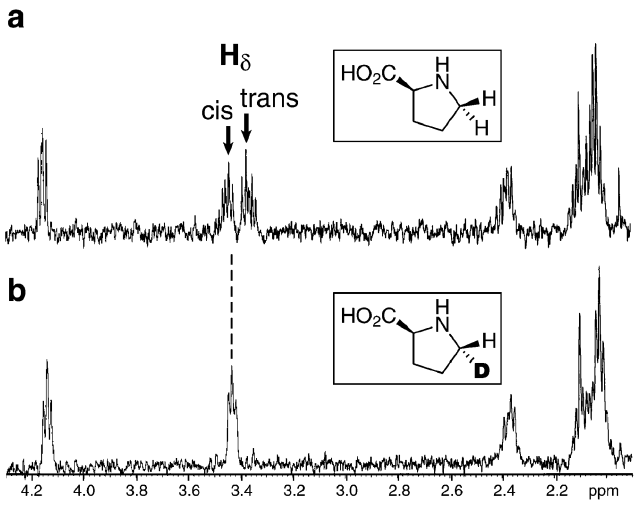


FIGURE 2. ^{1}H NMR spectra (500 MHz) of L-proline disclosing the stereospecificity of the NosF-mediated reduction with respect to coenzyme and substrate. (a) Nondeuterated L-proline from reaction of L-P5C (**21**) with NosF and A-side NADD, and (b) (2*S*,5*R*)-5-^{2}H-proline (= trans) from reaction with NosF and B-side NADD.

When NosF was incubated with DL-P5C (**21**/**22**) and B-side NADD, the aromatic region of the ^{1}H NMR spectrum of the crude product mixture exhibited the complete set of signals for the pyridine ring of NAD^+, suggesting that a deuteride had been transferred (Scheme 3). Indeed the isolated L-proline displayed a ^{1}H NMR spectrum that lacked the signal for one δ-proton (see Figure 2b). Comparison with L-proline from reaction with A-side NADD at the same pH (~5) demonstrated that the upfield signal of the δ-protons was missing (Figure 2), which is known to arise from the proton trans to the carboxyl group in proline.[22] The presence of a deuteron at this position was confirmed by ^{2}H NMR spectroscopy and the signal assignment in the ^{1}H NMR spectrum was confirmed by 1D NOE experiments.[23]

In summary, NosF was shown to be B-side specific, i.e., to transfer the (4*pro*-*S*)-hydrogen of NADH. Furthermore, it was demonstrated that NosF mediates the hydride transfer to the face of the pyrroline ring that is opposite to the carboxyl group in the substrate.

Stereospecificity of NAD^+ Oxidation by NosE. As with NosF, the stereospecificity of the nicotinamide oxidation by NosE was ascertained based on reactions with the nonmethylated substrate. Our strategy required the dideuterated (*S*)-Hava **30** that is easily accessible via the route described in Scheme 2, but by using $LiAlD_4$ in the reduction of methyl ester **3**. Incubation with NosE and NAD^+ under established conditions would result in generation of either A-side or B-side NADD, which are diastereomers and thus distinguishable by ^{1}H NMR spectroscopy.[21] Alternatively, the NosE assay could be coupled with an NosF assay and the final product L-proline isolated and its ^{1}H NMR spectrum inspected. Since NosF is known to be B-side specific, L-proline would contain two deuterium atoms at its δ carbon if NosE were B-side specific as well. Only one deuterium atom would be found if NosE had the opposite stereospecificity, i.e., were A-side specific (Scheme 4). Due to the instability of NADH/NADD but ease of proline isolation, the latter alternative was selected. The coupled assay was run in NosE buffer at pH 10; under these conditions NosF was active for only a few minutes. Because the L-proline subsequently isolated contained only one deuterium atom, NosE had to be A-side specific (Figure 3a). The deuterium label was cis to the carboxyl group since the deuterium must have been present in the intermediate (Scheme 4) and NosF transferred a hydride trans to the carboxyl group (compare Figure 3, parts a and b). The potential risk of scrambling if two NAD(H)-dependent enzymes of opposite stereospecificity are coupled was not observed, as NosF showed activity only for a short time period as mentioned. There was no need to remove or denature NosE prior to NosF addition.

To determine whether the 5*pro*-*R* or the 5*pro*-*S* hydrogen is abstracted in the NosE-catalyzed oxidation step, a stereoselective synthesis of either C-5 monodeuterated (2*S*)-Hava or (2*S*,4*S*)-5-hydroxyleucine was required. Again, this study was undertaken employing the nonmethylated substrate. Stereospecifically deuterated substrates as outlined in Scheme 5 were synthesized starting from *N*-benzyloxycarbonyl-L-glutamic acid (**31**), which was reacted with paraformaldehyde to give oxazolidinone acid **32**.[24] Action of oxalyl chloride on compound **32** yielded the acyl chloride **33**, which was converted to the deuterated aldehyde **34** via a modified Rosenmund reduction.[25] The two protecting groups on the amino functionality prevented an intramolecular cyclization. The stable aldehyde **34** was then subjected to Midland reduction,[26] i.e., reduction with (*R*)- or (*S*)-Alpine borane, to yield the monodeuterated compounds **35** and **36**, respectively. It has been established that (*R*)-Alpine borane gives the (*S*)-alcohol while (*S*)-Alpine borane gives the (*R*)-alcohol, and no exceptions are reported.[27] Acid hydrolysis[28] liberated the stereospecifically labeled target compounds **37** and **38** which were subsequently used in coupled assays with NosE and NosF. The ^{1}H NMR spectra of the isolated prolines (Figure 4) indicated not only the stereospecificity of NosE with respect to the substrate, but also that the Midland reduction proceeded in a highly stereoselective fashion. (*S*)-Alcohol **37** was converted to regular (nondeuterated) L-proline (Figure 4a), while (*R*)-alcohol **38** reacted to monodeuterated L-proline with the deuterium cis to the carboxyl group (Figure 4b), as when dideuterated Hava **30** was used (Figure 3a). The fact that deuterium was retained during the reaction sequence clearly indicated that NosE is *pro*-*S* specific with respect to the substrate.

(22) (a) Pogliani, L.; Ellenberger, M.; Valat, J. *Org. Magn. Reson.* **1975**, *7*, 61−71. (b) Altman, L. J.; Silberman, N. *Anal. Biochem.* **1977**, *79*, 302−309. (c) Anteunis, M. J. O.; Borremans, F. A. M.; Becu, C.; Sleeckx, J. *Int. J. Peptide Protein Res.* **1979**, *14*, 445−450.

(23) For standard proline at identical pH, irradiation of the α-proton shows an NOE to the upfield δ-proton, indicating that the latter proton is located trans to the carboxyl group.

(24) (a) Baldwin, J. E.; Lee, E. *Tetrahedon* **1986**, *42*, 6551−6554. (b) Lee, B. H.; Miller, M. J. *Tetrahedron Lett.* **1984**, *25*, 927−930.

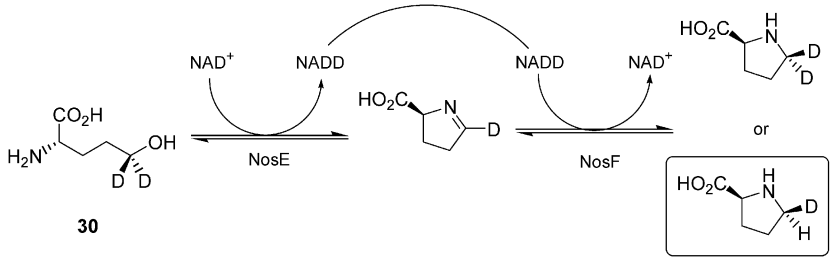
(25) Burgstahler, A. W.; Weigel, L. O.; Shaefer, C. G. *Synthesis* **1976**, 767−768.

(26) Midland, M. M.; Greer, S.; Tramontano, A.; Zderic, S. A. *J. Am. Chem. Soc.* **1979**, *101*, 2352−2355.

(27) Prabhakaran, P. C.; Gould, S. J.; Orr, G. R.; Coward, J. K. *J. Am. Chem. Soc.* **1988**, *110*, 5779−5784.

(28) Bold, G.; Steiner, H.; Moesch, L.; Walliser, B. *Helv. Chim. Acta* **1990**, *73*, 405−410.

SCHEME 4. Coupled NosE/NosF Assay To Determine the Stereospecificity of the NAD$^+$ Oxidation by NosE Prior to NosF Addition



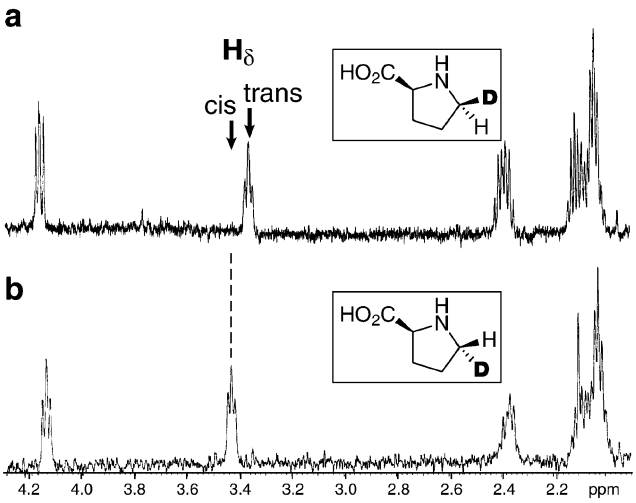


FIGURE 3. ^{1}H NMR spectra (500 MHz) of L-proline disclosing the stereospecificity of the NosE-mediated oxidation with respect to the coenzyme: (a) (2*S*,5*S*)-5-^{2}H-proline (= cis), the reaction product of the coupled assay starting from dideuterated (*S*)-Hava **30**, and (b) (2*S*,5*R*)-5-^{2}H-proline (= trans), the reaction product from reaction with NosF and B-side NADD (see Figure 2b) for comparison.

In summary, NosE is A-side specific with respect to the coenzyme, i.e., it mediates the hydride transfer from the substrate to the *re*-face of the pyridine moiety of NAD$^+$. This finding is consistent with the prediction that zinc-dependent dehydrogenases[29] or metal-dependent dehydrogenases in general[30] are *pro-R* specific. Furthermore, NosE catalyzes specifically the abstraction of the 5*pro-S* hydrogen of the substrate.

Conclusion

The characterization of two enzymes involved in the biosynthesis of 4-methylproline (MePro) in cyanobacteria has been described in this paper. The biosynthetic pathway leading to MePro has parallels in proline (Pro) biosynthesis, yet also displays some substantial differences. The intermediates in both pathways are structurally related: MeP5C for MePro and P5C for Pro. The high homology of NosF to other P5C reductases and the relaxed substrate specificity of NosF, also accepting L-P5C (**21**), might suggest that the *nosF* gene originated by duplication of the gene encoding the last step of the primary Pro anabolism. Recently, the biosynthesis of 3-methylproline in the paraherquamide family of fungal metabolites has been investigated and shown to proceed via a similar intermediate.[10] The second to last enzymatic steps in MePro and Pro biosynthesis are quite different. While glutamic acid γ-semialdehyde is commonly generated by two-electron reduction of the ω-carboxyl functionality of glutamic acid in Pro biosynthesis,[11] the corresponding C-4 methylated γ-semialdehyde (Scheme 1) originates from an oxidation step.

The relaxed substrate specificity of NosE and NosF may suggest that the specific incorporation of MePro into nostopeptolides A resides in the specificity of the NRPS module. Alternatively, specific incorporation of MePro may be governed by the reaction preceding NosE oxidation. Further studies are required to investigate the earlier step(s) in MePro biosynthesis.

Experimental Section

General. *E. coli* BL21(DE3)pLysS (Stratagene, La Jolla, CA) was used as an expression host and pRSETB (Invitrogen, Carlsbad, CA) as an expression vector. ^{1}H NMR spectra were recorded at 300 or 500 MHz, and ^{13}C NMR spectra at 125 MHz.

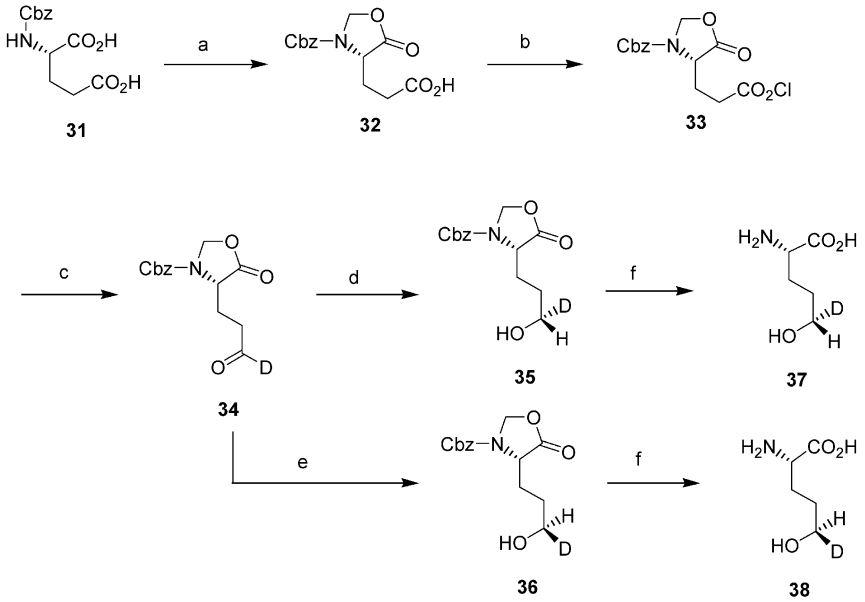
Incorporation of [1-^{13}C]L-leucine into nostopeptolide A1. The labeled amino acid (120 mg) was fed to a 20-L culture in eight equal doses starting from day 12 after inoculation. The culture was harvested on day 30 and the cyanobacterium lyophilized to give 10 g of freeze-dried material from which 1 mg of labeled nostopeptolide A1 was isolated. The ^{13}C NMR spectrum showed equally enhanced singlet signals (1%) at 175.3 (C-1 of Leu unit), 206.0 (C-3 of LeuAc unit), and 174.7 ppm (C-1 of MePro unit).

DNA Isolation, Manipulations, and Sequencing. In vitro DNA manipulations, cloning, and transformation of competent *E. coli* BL21(DE3)pLysS cells were performed according to Sambrook et al.[31] DNA sequencing was performed using the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit with Ampli*Taq*DNA Polymerase, FS (PE Applied Biosystems, Foster City, CA) according to the manufacturer's protocol and analyzed on a DNA sequencer. DNA sequences were aligned with the LASERGENE program Seqman (DNASTAR, London, United Kingdom).

PCR Amplification and Cloning of *nosE* and *nosF* for Overexpression. The coding sequences for NosE and NosF[6] were amplified by PCR using *Pfu* polymerase (Stratagene), primers with 5′ tails encoding restriction sites, and in-frame stop codons (Operon, Alameda, CA) and cosmid pREMc4 as DNA template.[6] The oligonucleotide sequences were as follows (5′ tails are underlined and restriction sites are in boldface): *nosE*For[*Bam*HI, *Nde*I], 5′-G**GGATCC**A**CAT ATG** CCC TTA GCA GCT GTG ATG AC-3′; *nosE*Rev[*Pst*I], 5′-G**CTGCAG** TCA AGA TAG ATT AGG TTG AAT CAC AG-3′; *nosF*For[*Bam*HI,

(29) Schneider-Bernlöhr, H.; Adolph, H.-W.; Zeppezauer, M. *J. Am. Chem. Soc.* **1986**, *108*, 5573−5576.
(30) Glasfeld, A.; Benner, S. A. *Eur. J. Biochem.* **1989**, *180*, 373−375.
(31) Sambrook, J.; Fritsch, E. F.; Maniatis, T. *Molecular Cloning, a Laboratory Manual*, 2nd ed.; Cold Spring Harbor Laboratory Press: Cold Spring Harbor, NY, 1989.

SCHEME 5. Stereoselective Synthesis of C-5 Monodeuterated (*S*)-Hava[a]



[a] Conditions: (a) paraformaldehyde, *p*-TsOH, toluene, reflux, 100 min, Dean−Stark apparatus; (b) $(COCl)_2$, cat. DMF, CH_2Cl_2, rt, 30 min; (c) D_2, 5% Pd/$BaSO_4$, THF, lutidine, rt, 2 h; (d) (*R*)-Alpine borane, THF, rt, 10 h; (e) (*S*)-Alpine borane, THF, rt, 10 h; (f) 5 N HCl, 100 °C, 2 h.

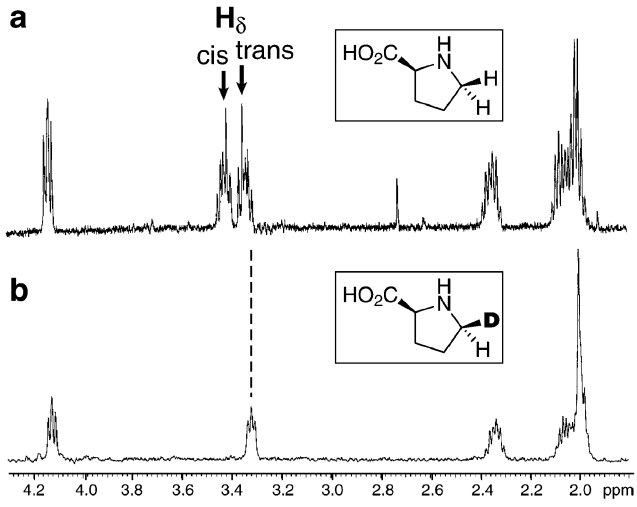


FIGURE 4. ^{1}H NMR spectra (500 MHz) of L-proline disclosing the stereospecificity of the NosE-mediated oxidation with respect to the substrate: (a) nondeuterated L-proline, the reaction product of the coupled assay starting from (2*S*,5*S*)-5-^{2}H-Hava (**37**), and (b) (2*S*,5*S*)-5-^{2}H-proline (= cis), the reaction product of the coupled assay starting from (2*S*,5*R*)-5-^{2}H-Hava (**38**).

*Nde*I], 5′-G**GGATCC**A**CAT ATG** CTC GAA GAT TTA CAA ATT GC-3′; *nosF*Rev[*Pst*I], 5′-G**CTGCAG** TTA ACT GAT ATT ACC TAA TTG TTG AG-3′.

The obtained PCR products were cloned using the PCR-Script Amp Cloning Kit (Stratagene), sequenced, and subcloned into the predigested (*Bam*HI/*Pst*I) expression vector pRSETB (Invitrogen). To verify the correct insert orientation (the pPCR-Script AMP SK(+) multiple cloning site contains additional *Bam*HI and *Pst*I sites), the recombinant expression plasmids were sequenced with the following primer: RSET, 5′-CTC ACT ATA GGG AGA CCA CAA C-3′.

Overexpression and Purification of *N*-His$_6$-Tagged NosE and NosF. The recombinant pRSET derivatives designated pNosEx and pNosFx were used to transform competent cells of *E. coli* BL21(DE3)pLysS. Transformed *E. coli* BL21(DE3)pLysS were grown in LB medium at 37 °C in the presence of 50 μg/mL of carbenicillin, 34 μg/mL of chloramphenicol, and 2.5 mM betaine. Cultures were induced with 0.4−0.8 mM IPTG at an A_{600} of 0.5−0.7 and allowed to grow for an additional 2−4 h at 30 °C. Cells were harvested at 8000 × *g* (10 min) and resuspended in wash buffer (50 mM NaH_2PO_4, pH 8.0, 300 mM NaCl, 20 mM imidazole) containing 0.1 mM PMSF and Protease Inhibitor Cocktail for the purification of poly(Histidine)-tagged proteins according to the manufacturers protocol (Sigma, St. Louis, MO), sonicated on ice (4 times for 15 s each), and then centrifuged for 20 min at 12,000 × *g* and 4 °C. Crude lysates were applied to Ni-NTA columns (Qiagen, Valencia, CA), the columns were washed twice with wash buffer, and the His$_6$-tagged proteins were eluted with elution buffer (50 mM NaH_2PO_4, pH 8.0, 300 mM NaCl, 250 mM imidazole). The eluates were applied onto an equilibrated Econo-Pac 10DG desalting column (Bio-Rad), and the fusion proteins were eluted with buffer A (50 mM Tris, 1 mM EDTA, 5% glycerol, pH 7.3, filtered; 1 mM DTT added before use). Expression level and protein purity were analyzed by SDS-polyacrylamide electrophoresis using Brilliant Blue G-Colloidal stain (Sigma). Protein concentration was determined by the Bradford Protein assay[32] or Micro Bradford Protein assay (Bio-Rad, Hercules, CA) using bovine serum albumin as a standard.

NosE Assays. Typical reaction mixtures contained 100 mM glycine (pH 10), 2 mM β-NAD, substrate, 1 mM $ZnSO_4$, and approximately 3 μg of NosE (*N*-His$_6$-tagged). Typical reaction volumes were 500 μL. NADH formation was monitored spectrophotometrically (A_{340} increase) at 42 °C. Initial rates were dependent upon protein concentration and were fitted to the Michaelis−Menten equation using nonlinear regression.

NosF Assays. Typical reaction mixtures contained 200 mM Tris (pH 8), substrate, 0.2 mM β-NADH (β-NADPH), and approximately 5 μg of NosF (*N*-His$_6$-tagged). Reaction volumes were usually 1 mL. NAD(P)H consumption was monitored spectrophotometrically (A_{340} decrease) at 42 °C. Initial rates were dependent upon protein concentration and were fitted to the Michaelis−Menten equation using nonlinear regression.

(32) Bradford, M. M. *Anal. Biochem.* **1976**, *72*, 248−254.

For determination of NosF stereospecificity, several reactions using 0.64 mM DL-P5C (**21**/**22**) were conducted simultaneously at 42 °C. After the absorption at 340 nm had decreased and reached a plateau, the reaction mixtures were combined, treated with $CHCl_3$, and centrifuged. The aqueous solution was concentrated and typically applied to a Dowex-50W column (H^+ form, 1.4 × 25 cm). Elution was done with 1 N HCl. Proline-containing fractions were identified by ^{1}H NMR, concentrated to dryness, reconstituted in H_2O, and subjected to chiral HPLC [Chirex phase 3126 (D)-Penicillamine (4.6 × 250 mm), Phenomenex; 2 mM $CuSO_4$−MeCN (95:5); detection at 254 nm]. L-Proline eluted at t_R 11.5 min. No D-proline was detected (t_R of standard, 22.2 min). The proline-containing fraction was concentrated and the residue redissolved in 1 N HCl. To remove copper ions from the solutions, hydrogen sulfide gas was bubbled through the solution for approximately 1 min. After centrifugation, the supernatant was concentrated to dryness and taken up in the NMR solvent to determine the deuterium content and position.

For the determination of the specificity of NosF with respect to the stereochemistry at C-3 of L-MeP5C, assays using (3*S*,5*S*)-MeP5C (**23**) and (3*R*,5*S*)-MeP5C (**24**) were carried out (both slightly epimerized at C-3). MePro was isolated and stereochemically characterized similarly as described above by chromatography on Dowex-50W and chiral HPLC. (2*S*,4*S*)-MePro eluted at t_R 27.0 min. No (2*S*,4*R*)-MePro was found after any assay (t_R of standard, 25.0 min).

Coupled Assays. Coupled assays, performed at 42 °C, were carried out to determine the stereospecificity of NosE. The reaction mixture contained 100 mM glycine (pH 10), 3 mM deuterated Hava, 2 mM β-NAD, 1 mM $ZnSO_4$, and approximately 10 μg of NosE. After 5 h of incubation at 42 °C, approximately 10 μg of NosF was added, resulting in a decrease in absorption at 340 nm for the following 15 min. Proline was then isolated as described above (see NosF assay) and deuterium content and position determined by NMR.

Preparation of A-Side and B-Side NADD. A-side and B-side NADD were synthesized and purified similarly as described by Viola et al.[19]

Synthesis of NosE Substrates. Intermediates **3**−**8** and **11**−**14** were synthesized as described starting from L- or D-glutamic acid (**1** or **2**).[5,14] Those compounds were converted into putative NosE substrates as described below:

***tert*-Butyl (*S*)-5-Hydroxy-2-(*N*-(9-(9-phenylfluorenyl))-amino)pentanoate (9).** The synthesis was carried out similarly as described by Koskinen and Rapoport.[14] Compound **3** (56.4 mg, 0.12 mmol) was dissolved in 3 mL of THF and cooled to −78 °C. Lithium aluminum hydride (11.4 mg, 0.30 mmol) was added. After 4 h of stirring at this temperature, 100 μL of saturated aqueous Na_2SO_4 was added to quench the reaction mixture. The mixture was allowed to warm to room temperature, at which an additional 5 mL of saturated aqueous Na_2SO_4 was added. The product was extracted into EtOAc (4 × 10 mL), and the combined organic layers were dried over $MgSO_4$, filtered, and evaporated. The crude product mixture was subjected to flash silica gel chromatography. Elution was initiated with 10% EtOAc in hexanes, and the product **9** eluted with 20% EtOAc in hexanes (40.5 mg, 76%): R_f 0.12 (20% EtOAc in hexanes); ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.19 (s, 9H), 1.47 (m, 1H), 1.53 (m, 2H), 1.55 (m, 1H), 2.52 (dd, *J* = 6.5, 4.5 Hz, 1H), 3.55 (br t, *J* ∼ 6.5 Hz, 2H), 7.18−7.70 (m, 13H).

***tert*-Butyl (2*R*)-5-Hydroxy-2-(*N*-(9-(9-phenylfluorenyl))-amino)pentanoate (10).** Compound **4** was converted analogously to **10** (35.8 mg, 68%). For NMR data, see compound **9**.

(2*S*,4*S*)-5-Hydroxyleucine (17). Compound **11** (22.5 mg, 52.4 μmol) was dissolved in 2.5 mL of a mixture of CH_2Cl_2−TFA (4:1) and stirred at room temperature for 16 h. The mixture was dried under N_2 and the residue partitioned between isooctane (5 mL) and H_2O (5 mL). The isooctane layer was back-extracted with 2 mL of H_2O, and the combined aqueous phases were evaporated. The residue was applied to a Dowex-50W cation-exchange resin (H^+ form, 1.2 × 5 cm) and washed with H_2O. Elution with 4 N NH_4OH and evaporation of the eluant gave **17** (6.5 mg, 84%): ^{1}H NMR (300 MHz, D_2O) δ 0.99 (d, *J* = 6.2 Hz, 3H), 1.74−1.88 (m, 3H), 3.47−3.53 (m, 2H), 3.77 (dd, *J* ∼ 10, 4 Hz, 1H).

(2*S*,4*R*)-5-Hydroxyleucine (18). Analogous treatment of 12.0 mg (27.1 μmol) of compound **12** yielded **18** (3.5 mg, 88%): ^{1}H NMR (300 MHz, D_2O) δ 1.00 (d, *J* = 6.7 Hz, 3H), 1.71 (m, 1H), 1.84 (m, 1H), 1.95 (m, 1H), 3.49 (dd, *J* = −11.1, 6.3 Hz, 1H), 3.53 (dd, *J* = −11.1, 5.7 Hz, 1H), 3.80 (t, *J* = 6.6 Hz, 1H).

(2*R*,4*R*)-5-Hydroxyleucine (19). Analogous treatment of compound **13** (15.0 mg, 33.8 μmol) yielded **19** (4.0 mg, 80%). For NMR data, see compound **17**.

(2*R*,4*S*)-5-Hydroxyleucine (20). Analogous treatment of compound **14** (12.0 mg, 27.1 μmol) yielded **20** (3.2 mg, 80%). For NMR data, see compound **18**.

(*S*)-5-Hydroxy-2-aminovaleric Acid (15). Analogous treatment of 30.0 mg (69.8 μmol) of compound **9** yielded **15** (8.5 mg, 91%): ^{1}H NMR (300 MHz, D_2O) δ 1.61 (m, 2H), 1.88 (m, 2H), 3.62 (t, *J* = 6 Hz, 1H), 3.74 (t, *J* = 6 Hz, 1H).

(*R*)-5-Hydroxy-2-aminovaleric Acid (16). Analogous treatment of 30.0 mg (69.8 μmol) of compound **10** yielded **16** (7.5 mg, 81%). For NMR data, see compound **15**.

***tert*-Butyl (*S*)-5-Hydroxy-2-(*N*-(9-(9-phenylfluorenyl))-amino)-5,5-dideuteriopentanoate.** Compound **3** (56.4 mg, 0.12 mmol) was reduced as described for the synthesis of **9**, except that lithium aluminum deuteride was used as the reducing agent, to afford the target compound (35.6 mg, 67%): R_f 0.12 (20% EtOAc in hexanes); ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.19 (s, 9H), 1.47 (m, 1H), 1.53 (m, 2H), 1.55 (m, 1H), 2.52 (dd, *J* = 6.6, 4.4 Hz, 1H), 7.18−7.70 (m, 13H).

(*S*)-5-Hydroxy-2-amino-5,5-dideuteriovaleric Acid (30). As described for the synthesis of **15** from **9**, *tert*-butyl (*S*)-5-hydroxy-2-(*N*-(9-(9-phenylfluorenyl))amino)-5,5-dideuteriopentanoate (35.5 mg, 8.2 μmol), generated in the previous step, was converted to **30** (10.9 mg, 98%). ^{1}H NMR (300 MHz, D_2O) δ 1.61 (m, 2H), 1.88 (m, 2H), 3.73 (t, *J* = 6 Hz, 1H).

The stereospecifically labeled NosE substrates were synthesized in the following manner:

(4*S*)-3-(Benzyloxycarbonyl)-4-(carboxyethyl)-1,3-oxazolidin-5-one (32). The reaction was carried out analogously as described for the aspartic acid derivative.[24a] To a suspension of *N*-Cbz-L-glutamic acid (**31**) (2.16 g, 7.7 mmol) in 150 mL of toluene were added paraformaldehyde (1.46 g) and *p*-TsOH (154 mg). The mixture was heated under reflux for 100 min using a Dean−Stark apparatus for azeotropic removal of water. The product mixture was filtered through silica gel and the product eluted with Et_2O (400 mL). The solvent was evaporated to yield **32** as a colorless oil (2.25 g, 100%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 2.10−2.60 (m, 4H), 4.38 (t, *J* = 6 Hz, 1H), 5.10−5.30 (m, 3H), 5.45 (br, 1H), 7.30−7.40 (m, 5H).

(4*S*)-3-(Benzyloxycarbonyl)-4-(3-chloro-3-oxopropyl)-1,3-oxazolidin-5-one (33). A solution of oxazolidinone acid **32** (2.26 g, 7.7 mmol) in CH_2Cl_2 (7.7 mL) was treated with redistilled oxalyl chloride (1.04 mL, 11.7 mmol) at room temperature. One drop of anhydrous DMF was added as catalyst. After 30 min the solvent and the excess reagent were removed under water aspirator vacuum and DMF under high vacuum to give **33** as an off-white solid residue (2.40 g, 100%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 2.10−2.40 (m, 4H), 3.07 (m, 2H), 4.34 (t, *J* = 6 Hz, 1H), 5.10−5.30 (m, 3H), 5.45 (br, 1H), 7.30−7.40 (m, 5H).

(4*S*)-3-(Benzyloxycarbonyl)-4-(3-deuterioformylethyl)-1,3-oxazolidin-5-one (34). Acyl chloride **33** (1.58 g, 5.0 mmol) was dissolved in 4 mL of THF and added over 3 min to the deuterium-equilibrated catalyst (5% Pd/$BaSO_4$, 0.30 g) in 20 mL of THF containing 1 equiv of redistilled lutidine (0.58 mL, 5.0 mmol). After being stirred for 2 h at room temperature, the mixture was filtered. The filtrate was diluted with Et_2O (60 mL) and washed with dilute HCl (pH 3, 20 mL), H_2O (20 mL), saturated $NaHCO_3$ (2 × 20 mL), H_2O (20 mL), and saturated NaCl (20 mL). The organic phase was dried ($MgSO_4$), and the solvent evaporated to yield aldehyde **34** (0.68 g, 48%).

^{1}H NMR (300 MHz, $CDCl_3$) δ 2.1−2.7 (m, 2H), 4.37 (t, J = 6 Hz), 5.05−5.25 (m, 3H), 5.50 (br, 1H), 7.30−7.43 (m, 5H); ^{2}H NMR (77 MHz, $CHCl_3$) δ 9.70.

(4*S*)-3-(Benzyloxycarbonyl)-4-[(3*S*)-3-hydroxy-3-deuteriopropyl]-1,3-oxazolidin-5-one (35). Aldehyde **34** (0.322 g, 1.16 mmol) was dissolved in 3 mL of THF and added via syringe to a 0.5 M solution of (*R*)-Alpine borane in THF (2.78 mL, 1.38 mmol) under N_2 atmosphere and while stirring. The mixture was stirred overnight, and then THF was removed by water aspirator vacuum at 35 °C bath temperature. The residue was redissolved in Et_2O (20 mL) and cooled in an ice bath. Ethanolamine (84.2 mg, 1.38 mmol) was added and stirring continued for 20 min on ice. The precipitate was removed after centrifugation, and the supernatant was concentrated. The crude product was subjected to flash silica gel chromatography (EtOAc−hexanes 1:1) to give the (*S*)-alcohol **35** as a colorless oil (59.7 mg, 18%). R_f 0.25 (EtOAc−hexanes 1:1); ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.40−2.30 (m, 4H), 3.60 (m, 1H), 4.36 (m, 1H), 5.10−5.25 (m, 3H), 5.50 (br, 1 H), 7.30−7.40 (m, 5H); ^{2}H NMR (77 MHz, $CHCl_3$) δ 3.60.

(4*S*)-3-(Benzyloxycarbonyl)-4-[(3*R*)-3-hydroxy-3-deuteriopropyl]-1,3-oxazolidin-5-one (36). Compound **36** was synthesized from **34** in the same manner as described for **35**, except that (*S*)-Alpine borane was used. (*R*)-Alcohol **36** was obtained as a colorless oil (62.4 mg; 19%). R_f 0.25 (EtOAc−hexanes 1:1). The NMR spectra of **35** and **36** were indistinguishable.

(2*S*,5*S*)-5-Hydroxy-5-deuterio-2-aminovaleric acid (37). Compound **35** (30 mg, 0.11 mmol) was incubated with 5 N HCl (1.5 mL) at 100 °C for 2 h. The reaction mixture was extracted with Et_2O (2 × 1.0 mL). The combined organic phases were back-extracted with H_2O (1.0 mL) and the combined aqueous phases concentrated to dryness. The crude product was redissolved in a minimal amount of H_2O and applied to a Dowex-50W (H^+ form) column. After washing with H_2O the product was eluted with 4 N NH_4OH (11.2 mg, 78%). ^{1}H NMR (500 MHz, D_2O) δ 1.61 (m, 2H), 1.90 (m, 2H), 3.62 (t, J = 6.1 Hz, 1H), 3.74 (t, J = 6.0 Hz, 1H); ^{2}H NMR (77 MHz, H_2O) δ 3.62.

(2*S*,5*R*)-5-Hydroxy-5-deutero-2-aminovaleric acid (38). Compound **38** was generated from **36** as described for **37**. (*R*)-Alcohol **38** was obtained as a colorless, amorphous solid (13.1 mg; 91%). ^{1}H NMR (500 MHz, D_2O) δ 1.64 (m, 2H), 1.93 (m, 2H), 3.65 (t, J = 6.1 Hz), 3.79 (t, J = 6.0 Hz); ^{2}H NMR (77 MHz, H_2O) δ 3.64.

Synthesis of NosF Substrates. Syntheses under aqueous conditions yielded the following imines while oxidation in organic solvents (attempted in the case of the methylated substrates) resulted in enamine formation:

DL-P5C (21/22). Racemic P5C was generated and purified by cation-exchange chromatography similarly as described.[16] The P5C concentration was determined by reaction with *o*-aminobenzaldehyde (*o*-AB) and spectrophotometrical measurement of the absorption of the adduct at 444 nm (ϵ_{444} = 2940 M^{-1} cm^{-1}).[16a] Common isolated yields of DL-P5C (**21**/**22**) were 70−80% (∼2.5 mM solutions).

L-P5C (21). Compound **21** was synthesized similarly as described for α-aminoadipic-δ-semialdehyde.[17] (*S*)-Hava (**15**) (4 mg) was incubated with CrO_3 (3 mg) and 4 N HCl (0.5 mL) at 80 °C for 1 h. The reaction mixture was carefully neutralized with NaOH solutions, and $Cr(OH)_3$ was precipitated by centrifugation. The supernatant was acidified (1 N HCl) to pH <2 and stored at 4 °C. Purification could be achieved by cation-exchange chromatography (Dowex-50W, H^+ form), but the crude product was also usable in subsequent NosF assays. L-P5C concentration was determined as above. When the crude product was used, after mixing with Tris buffer (pH 8), the additional $Cr(OH)_3$ that had formed had to be removed by centrifugation prior to NADH and enzyme addition.

D-P5C (22). Compound **22** was generated in the same manner as L-P5C (**21**) but using D-Hava (**16**).

(3*S*,5*S*)-3-Methyl-P5C (23). The synthesis was carried out as described above for L-P5C (**21**), starting from (2*S*,4*S*)-5-hydroxyleucine (**17**). However, some epimerization at C-3 will have occurred (see text). The MeP5C concentration was estimated by color reaction with *o*-AB, assuming that ϵ_{444} of the adduct corresponded closely to the adduct of P5C with *o*-AB. UV spectra were found to exhibit the same maximum at ∼444 nm.

(3*R*,5*S*)-3-Methyl-P5C (24). Analogous oxidation of (2*S*,4*R*)-5-hydroxyleucine (**18**) yielded compound **24** and generated minor amounts of its C-3 epimer (see text). The concentration of MeP5C was estimated as described above.

The synthesis of enamine **29** is detailed elsewhere.[18]

Acknowledgment. Funding was provided by R01 grant CA12623 from the National Cancer Institute. Thanks to John Raycraft and Cheryl Phillipson for support in protein overexpressions and running enzyme assays, to Wesley Y. Yoshida for the acquisition of many NMR spectra, and to Dr. Sreedhara Chaganty for technical assistance in isolation and synthesis.

JO026479Q