Dichlorination and Bromination of a Threonyl-S-Carrier Protein by the Non-heme Fe\(^{II}\) Halogenase SyrB2

Frédéric H. Vaillancourt,[a, b] David A. Vosburg,[a, c] and Christopher T. Walsh*[a]  

Biosynthetic tailoring of nonribosomal peptide and polyketide natural products can enhance their biological activities.[1–3] Tai-

nationally, biosynthetic gene clusters; this sug-

gests a tailoring role in specific natural product assembly. This

can be found embedded in biosynthetic gene clusters; this sug-

gests a tailoring role in specific natural product assembly. This

can be found embedded in biosynthetic gene clusters; this sug-

gests a tailoring role in specific natural product assembly. This

can be found embedded in biosynthetic gene clusters; this sug-

gests a tailoring role in specific natural product assembly. This

can be found embedded in biosynthetic gene clusters; this sug-

gests a tailoring role in specific natural product assembly. This

can be found embedded in biosynthetic gene clusters; this sug-

gests a tailoring role in specific natural product assembly. This

can be found embedded in biosynthetic gene clusters; this sug-

gests a tailoring role in specific natural product assembly. This

can be found embedded in biosynthetic gene clusters; this sug-

gests a tailoring role in specific natural product assembly. This

can be found embedded in biosynthetic gene clusters; this sug-

gests a tailoring role in specific natural product assembly. This

can be found embedded in biosynthetic gene clusters; this sug-

gests a tailoring role in specific natural product assembly. This

can be found embedded in biosynthetic gene clusters; this sug-

gests a tailoring role in specific natural product assembly. This

can be found embedded in biosynthetic gene clusters; this sug-

gests a tailoring role in specific natural product assembly. This

can be found embedded in biosynthetic gene clusters; this sug-

gests a tailoring role in specific natural product assembly. This

can be found embedded in biosynthetic gene clusters; this sug-

gests a tailoring role in specific natural product assembly. This

can be found embedded in biosynthetic gene clusters; this sug-

gests a tailoring role in specific natural product assembly. This

can be found embedded in biosynthetic gene clusters; this sug-

gests a tailoring role in specific natural product assembly. This

can be found embedded in biosynthetic gene clusters; this sug-

gests a tailoring role in specific natural product assembly. This

can be found embedded in biosynthetic gene clusters; this sug-

gests a tailoring role in specific natural product assembly. This

can be found embedded in biosynthetic gene clusters; this sug-

gests a tailoring role in specific natural product assembly. This

can be found embedded in biosynthetic gene clusters; this sug-

gests a tailoring role in specific natural product assembly. This

can be found embedded in biosynthetic gene clusters; this sug-

gests a tailoring role in specific natural product assembly. This

can be found embedded in biosynthetic gene clusters; this sug-

gests a tailoring role in specific natural product assembly. This

can be found embedded in biosynthetic gene clusters; this sug-

gests a tailoring role in specific natural product assembly. This

can be found embedded in biosynthetic gene clusters; this sug-

gests a tailoring role in specific natural product assembly. This

can be found embedded in biosynthetic gene clusters; this sug-

gests a tailoring role in specific natural product assembly. This

can be found embedded in biosynthetic gene clusters; this sug-

Natural products can enhance their biological activities.[1–3] Tai-

nationally, biosynthetic gene clusters; this sug-

gests a tailoring role in specific natural product assembly. This

can be found embedded in biosynthetic gene clusters; this sug-

gests a tailoring role in specific natural product assembly. This

can be found embedded in biosynthetic gene clusters; this sug-

gests a tailoring role in specific natural product assembly. This

can be found embedded in biosynthetic gene clusters; this sug-

gests a tailoring role in specific natural product assembly. This

can be found embedded in biosynthetic gene clusters; this sug-

gests a tailoring role in specific natural product assembly. This

can be found embedded in biosynthetic gene clusters; this sug-

gests a tailoring role in specific natural product assembly. This

can be found embedded in biosynthetic gene clusters; this sug-

gests a tailoring role in specific natural product assembly. This

can be found embedded in biosynthetic gene clusters; this sug-

gests a tailoring role in specific natural product assembly. This

can be found embedded in biosynthetic gene clusters; this sug-

gests a tailoring role in specific natural product assembly. This

can be found embedded in biosynthetic gene clusters; this sug-

gests a tailoring role in specific natural product assembly. This

can be found embedded in biosynthetic gene clusters; this sug-

gests a tailoring role in specific natural product assembly. This

can be found embedded in biosynthetic gene clusters; this sug-

gests a tailoring role in specific natural product assembly. This

can be found embedded in biosynthetic gene clusters; this sug-

gests a tailoring role in specific natural product assembly. This

can be found embedded in biosynthetic gene clusters; this sug-

gests a tailoring role in specific natural product assembly. This

can be found embedded in biosynthetic gene clusters; this sug-

gests a tailoring role in specific natural product assembly. This

can be found embedded in biosynthetic gene clusters; this sug-

gests a tailoring role in specific natural product assembly. This

can be found embedded in biosynthetic gene clusters; this sug-

gests a tailoring role in specific natural product assembly. This

can be found embedded in biosynthetic gene clusters; this sug-

gests a tailoring role in specific natural product assembly. This

The Fe\(^{II}\) halogenases represent a new branch of the O\(_2\)- and

\(\alpha\)-ketoglutarate-decarboxylating superfamily and are powerful

enough to halogenate unactivated carbon centers on aminoa-

cyl groups tethered to nonribosomal peptide synthetase assem-

bly lines.[10,11] Thus, the 4-Cl-

l

S

-threonine in the amino acid coronamic acid arises by a similar

mediated chlorination is cryptic in cyclopropane formation.[11]

In surveying natural products in which biological chlorina-

tion is likely to have occurred at an unactivated carbon center,

the remarkable functionalization of the two prochiral methyl

groups of leucine to yield the regio- and stereospecific genera-

tion of a trichloromethyl group in the biosynthesis of the cya-

nobacterial metabolites barbamide (2), dysidenin (3), and dys-

deathiazole (4; Scheme 2A) suggests analogies with the above

Fe\(^{II}\) halogenases.[13,14] Indeed, the barbamide biosynthetic gene

cluster has been sequenced and contains two genes (barB1 and

barB2) that encode proteins homologous to SyrB2, but no activity

has yet been reported.[13] barB1 and barB2 homologues have also

been found in the dysidenin and dysideathiazole producers (dysB1/dysB2; one pair in each producer).[13] Only a few natural products contain bromine where biological bromi-

teotide linkage on the peptidyl carrier protein domain of its

partner protein SyrB1. 4-Cl-threonyl-S-SyrB1 was gently hydro-

lyzed by addition of the thioesterase TycF and detected as the

isoidole adduct (Figure 1A). With an almost equimolar

ratio of SyrB2/SyrB1, SyrB2 generated a new peak (Figure 1B)

that coeluted with the isoidole derivative of authentic 4,4-

diCl-Thr, synthesized as noted in the Experimental Section.

Mass analysis of the new enzymatic product confirmed both

the mass and isotope ratios of the diCl-L-Thr isoidole deriva-

tive (calcd for [M+H]\(^{+}\) = 392.0 (100%), 394.0 (71%); found 391.7

(100%), 394.1 (68%)). In addition, the ratio of the relative in-

tensity of the peaks corresponding to 4-Cl-L-Thr and 4,4-diCl-L-

Thr for the reaction run in the presence of [\(^{38}\)Cl] \(^{-}\) is doubled in the

radioactivity detection channel (Figure 1B, trace c) when

compared to the UV channel (Figure 1B, trace d) of the same

reaction; this is in good agreement with the presence of two

chloro substituents. Because the substrate for SyrB2 is a coval-

tent aminoacyl-S protein and the chlorinated product(s) remain

covalently tethered, kinetic analysis would be challenging.

However, the released mono- and diCl-L-Thr products were ob-

tained at a maximum ratio of about 0.38:1 diCl-L-Thr/Cl-L-Thr

(Figure 1C). When comparing the ratio of diCl-L-Thr/Cl-L-Thr in

Figure 1C and B, it can be seen that the ratio is higher for

panel B (1:1 diCl-L-Thr/Cl-L-Thr). This seems to be due to the

difference in initial oxygen concentration prior to SyrB2 addi-

tion. In panel B, the reaction mixture was anaerobic prior to

SyrB2 addition, whereas the buffer was air-saturated for

panel C. The slow addition of oxygen after SyrB2 addition in-

creases the product yield, as shown in Figure 1B. This was also

observed when demonstrating the oxygen dependency of the

reaction in a prior study (Figure 2B in ref.[10]), in which full


[a] Dr. F. H. Vaillancourt,* Prof. Dr. D. A. Vosburg,* Prof. C. T. Walsh
Department of Biological Chemistry and Molecular Pharmacology
Harvard Medical School, 240 Longwood Avenue, Boston, MA 02115 (USA)
Fax: (+1) 617-432-0438  
E-mail: christopher.walsh@hms.harvard.edu
[b] Dr. F. H. Vaillancourt*
Present address: Department of Biological Sciences
Research and Development, Boehringer Ingelheim (Canada) Ltd  
Laval, QC, H7S 2G5 (Canada)
[c] Prof. Dr. D. A. Vosburg
Present address: Department of Chemistry, Harvey Mudd College
Claremont, CA 91711 (USA)
[*] These authors contributed equally to this work.
turnover to 4-Cl-L-Thr was observed at low SyrB2/SyB1 enzyme ratios.

Inside the syringomycin-producing _Pseudomonas syringae_ bacterial cell, the eight-module SyrE protein would constitute the rest of the assembly line to capture 4-Cl-L-Thr and install it as residue nine in the nonadepsipeptide natural product. In this in vitro assay with purified SyrB1 and SyrB2, the monochlorothreonyl moiety stays tethered on the PCP domain of SyrB1; this allows SyrB2 to iteratively cleave the second C–H bond at carbon 4 and form an additional C–Cl bond. The absence of the dichlorinated Thr analogue of syringomycin in vivo could be due to a specific and rapid transfer of 4-Cl-L-Thr from SyrB1 to the SyrE protein, potentially performed by SyrC, a CmaE homologue, and/or to the narrow specificity of the last condensation domain of SyrE, which catalyzes the incorporation of the ninth amino acid (4-Cl-L-Thr) in the syringomycin peptide.

SyrB2 was also shown to brominate L-Thr-S-SyrB1 in reactions performed with excess sodium bromide (Figure 2A). The new major peak coeluted with the isoindole derivative of authentic 4-Br-L-Thr, synthesized as noted in the Experimental Section. Mass analysis of the new enzymatic product confirmed both the mass and isotope ratios for the Br-L-Thr isoindole derivative (Figure 2B). The brominating activity of SyrB2 correlates well with the observation that bromosyringomycin E is formed in vivo when the producing strain is grown in the presence of excess sodium bromide. A small amount of 4-Cl-L-Thr was also produced; this is due to the presence of a small amount of chloride in the buffer (∼10 μM from the HEPES solid). It is known that an equimolar amount of chloride is present in the SyrB2 enzyme preparation. As 20 μM SyrB2 was used, the total chloride concentration was therefore 30 μM. In comparison, 60 μM bromide was present in the reaction mixture. By assuming free exchange of bromide with chloride in

Scheme 1. Biosynthesis of 4-Cl-L-Thr, a key component of syringomycin E (1).

the enzyme prior to oxygen exposure, a preference for chloride versus bromide by a factor of 180 can be estimated by comparing the peak intensities. Another peak (peak 2), distinct from the remaining L-Thr, was also detected. Mass spectrometric analysis proved that this peak has an identical mass to a γ-lactone derivative of the Br-L-Thr isoindole compound (Figure 2B). Formation of this lactone is an artifact of the derivatization procedure that is performed at pH 8.0, as this side product is also observed when pure 4-Br-L-Thr is derivatized. The lactone is presumably formed by intramolecular displacement of bromide by the aminocyl carboxylate.

When the reaction was performed by adding SyrB2 to anaerobic L-Thr-SyrB1, and slowly adding oxygen by exposing the tubes to air outside of the glovebox. C) Analysis of the amount of L-Thr (●) consumed, 4-Cl-L-Thr (●) and 4,4-diCl-L-Thr (●) produced at different ratios of SyrB2/SyrB1. The concentration of SyrB1 was kept constant at 120 μM. All reactions in this panel were performed by adding SyrB2 to L-Thr-SyrB1 in air-saturated buffer.

Figure 1. Biosynthesis of 4,4-diCl-L-Thr by the SyrB2 halogenase. A) Procedure for the preparation and derivatization of amino acids for HPLC analysis. B) Traces of hydrolyzed amino acid obtained after incubation of L-Thr-SyrB1 with SyrB2 (0.7:1 SyrB2/SyrB1). Radio-HPLC traces of (a) the control reaction performed with L-[14C]Thr-SyrB1 in the absence of SyrB2; (b) the reaction performed with L-[14C]Thr-SyrB1, chloride, O2, α-ketoglutarate, and SyrB2; (c) the reaction performed with L-Thr-SyrB1, chloride, O2, α-ketoglutarate, and SyrB2 in presence of Na3Cl. d) UV trace (338 nm) of the same reaction (c). The 0.6 min shift between trace d (UV) and traces a, b and c (radioactivity) is typical and caused by the position of the radioactivity detector after the UV detector. All reactions in this panel were performed by adding SyrB2 to anaerobic L-Thr-SyrB1, and slowly adding oxygen by exposing the tubes to air outside of the glovebox. C) Analysis of the amount of L-Thr (●) consumed, 4-Cl-L-Thr (●) and 4,4-diCl-L-Thr (●) produced at different ratios of SyrB2/SyrB1. The concentration of SyrB1 was kept constant at 120 μM. All reactions in this panel were performed by adding SyrB2 to L-Thr-SyrB1 in air-saturated buffer.

Figure 2. Biosynthesis of 4-Br-L-Thr by the SyrB2 halogenase. A) Trace of hydrolyzed amino acid obtained after incubation of 83 μM L-Thr-SyrB1 with 60 mM bromide, O2, α-ketoglutarate, and 20 μM SyrB2. B) Mass spectrometric analysis of the isoindole derivative of 4-Br-L-Thr (together with its γ-lactone derivative) hydrolyzed from SyrB1.
formed under similar conditions with fluoride or iodide instead of bromide, no new products were formed.

These results directly establish that the non-heme Fe^{II} superfamily member SyrB2 can catalyze bromination and tandem chlorinations at the ω-position of the threonyl-S-protein substrate. The dichlorinating activity is a strong predictor that the cyanobacterial homologues, BarB1/BarB2 and DysB1/DysB2,[13,14] will iteratively chlorinate the pro-R unactivated methyl group of a leucyl-S-protein substrate all the way to a trichloromethyl group in the production of the cyanobacterial metabolites 2–4 (Scheme 2A). Whether non-heme Fe^{II} halogenases are involved in the biosynthesis of brominated natural products remains to be shown. However, results from the present study show that this is a possibility. The preference for chlorination so far observed in this class of enzymes could be due to the environment of the producing strain, in which chloride is more abundant than bromide. Detection of the ability of SyrB2 to act as a dichlorinating and brominating enzyme will set the stage for mechanistic analysis on both the timing of halogenation(s) and proposed homolytic C–H cleavages and subsequent rebound mechanisms for Cl^− transfer from high-valent oxoiron[10,11] species as the same carbon site is successively chlorinated.

**Experimental Section**

Chemical synthesis of 4,4-diCl-Thr: Dichloroacetaldehyde, which is not stable for prolonged storage, was prepared from diethyl acetal by the method of Lindholm et al.[15] A solution of lithium hexamethyldisilazide (LHMDS; 1 m, in THF; 23 mL, 2.3 mmol) was added to THF (3 mL) at −78 °C. A solution of Boc-Gly-OrBu (231 mg, 1 mmol) in THF (2 mL) was added dropwise. After 1 h at −78 °C, dichloroacetaldehyde (121 μL, 1.5 mmol) was added dropwise. After 45 min (still only ca. 30% conversion by TLC), the reaction was quenched with saturated NH₄Cl (7 mL), warmed to room temperature, and extracted with EtOAc (20 mL) (2×10 mL). The combined organic layers were washed with brine (1×10 mL), dried (MgSO₄), filtered, and concentrated in vacuo to give 0.5 g of a yellow oil. This oil was chromatographed on silica (10:1 hexanes/EtOAc, 2:1 hexanes/EtOAc) to provide Cbz-4-Br-Thr-OrBu (9.7 mg, 100% yield). 4-Br-Thr was conveniently stored as its hydrobromide salt. 1H NMR (500 MHz, CDCl₃): δ = 4.32 (d, J = 1.8 Hz, 1H), 4.15 (dd, J = 2.5, 4.0 Hz, 1H), 3.52 (dd, J = 2.5, 2.7 Hz, 1H), 3.22 (s, 9H), 1.49 (s, 9H), 1.46 (s, 9H).

Boc-4,4-diCl-Thr-OrBu (8 mg, 0.02 mmol) was dissolved in TFA over 20 min, but afforded Boc-4,4-diCl-Thr-OBzl (150 mg, 100% yield). 4-Br-Thr was also determined as previously described.[15,16] The mass of the released amino acids from SyrB1 were also determined as previously described.[17] The only altered parameters were the SyrB2-L-S-SyrB1 concentration and the use of NaBr instead of a chloride salt for the bromination experiments. To minimize chloride contamination, L-Thr-S-SyrB1 was desalted anaerobically in HEPES buffer (20 mM, pH 7.5), as previously described,[18] prior to incubation with NaBr, O₂, α-ketoglutarate and SyrB2. Details of the concentrations that are different from previously described assays are in the figure legends.

**Acknowledgements**

This work was supported in part by NIH grants GM 20011 and GM 49338 (C.T.W.), a Merck-sponsored Fellowship of the Helen Hay Whitney Foundation (F.H.V.), an NSERC Postdoctoral Fellowship (F.H.V.), and a Jane Coffin Childs Memorial Fund for Medical Research Fellowship (D.A.V.).

**Keywords:** amino acids · biosynthesis · enzymes · halogenation · natural products


Received: November 17, 2005
Published online on March 10, 2006