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

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Biosynthetic tailoring of nonribosomal peptide and polyketide natural products can enhance their biological activities.[1–3] Tailoring enzymes can introduce alkyl, acyl, or glycosyl groups onto natural product scaffolds and can oxidize or halogenate biosynthetic intermediates. Chlorinated and brominated molecules make up more than 95% of the more than 4500 known halogenated metabolites.[4] Chloro and bromo substituents are frequently found on aromatic and heteroaromatic rings, and many terpene scaffolds are also brominated and chlorinated by marine microorganisms.[4–6] Halogenating enzymes discovered to date fall into two categories based on their utilization either of hydrogen peroxide (haloperoxidases) or molecular oxygen (halogenases) as required oxidants.[7–11] Haloperoxidases can contain either heme iron or a vanadate cofactor, thought to generate enzyme-bound hapohalite equivalents as proximal OCl or OBr.[7,8] The O_2-utilizing halogenases are typically found embedded in biosynthetic gene clusters; this suggests a tailoring role in specific natural product assembly. This second class of enzymes uses either $FADH_2$ or non-heme Fe^{II} to activate chloride or bromide oxidatively.[9–11] The flavoproteins work on electron-rich aromatic and heteroaromatic substrates.[9,12]

The Fe^{II} halogenases represent a new branch of the O_2 and α-ketoglutarate-decarboxylating superfamily and are powerful enough to halogenate unactivated carbon centers on aminoacyl groups tethered to nonribosomal peptide synthetase assembly lines.[10,11] Thus, the 4-Cl-L-Thr residue in the phytotoxic lipodepsipeptide syringomycin E (**1**) is generated by the non-heme Fe^{II} halogenase SyrB2 (Scheme 1).[10] Chlorination occurs on the threonyl skeleton only while it is linked via a thioester to a peptidyl carrier protein domain. Remarkably, the cyclopropane ring in the amino acid coronamic acid arises by a similar γ-chlorination of an L-*allo*-Ile-*S*-protein by the halogenase CmaB.[11] The γ-chloride is then displaced intramolecularly by a thioester enolate by action of CmaC. Therefore, the CmaB-mediated chlorination is cryptic in cyclopropane formation.[11]

In surveying natural products in which biological chlorination 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 generation of a trichloromethyl group in the biosynthesis of the cyanobacterial metabolites barbamide (**2**), dysidenin (**3**), and dysideathiazole (**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).[14] Only a few natural products contain bromine where biological bromination might have occurred at an unactivated carbon site. One example is lyngbyaloside B (**5**; Scheme 2B),[15] which is also of cyanobacterial origin and could arise from bromination of an unactivated carbon on a biosynthetic precursor. Whether non-heme Fe^{II} halogenases are involved in the biosynthesis of brominated natural products is unknown. To evaluate whether the non-heme Fe^{II} family of enzymes can indeed carry out bromination and iterative chlorinations at the same carbon site, we have further examined the activity of SyrB2.

SyrB2 was shown to act on the threonyl group presented in thioester linkage on the peptidyl carrier protein domain of its partner protein SyrB1. 4-Cl-threonyl-*S*-SyrB1 was gently hydrolyzed by addition of the thioesterase TycF and detected as the isoindole adduct (Figure 1A).[10,16] With an almost equimolar ratio of SyrB2/SyrB1, SyrB2 generated a new peak (Figure 1B) that coeluted with the isoindole 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 isoindole derivative (calcd for $[M+H]^+$ 392.0 (100%), 394.0 (71%); found 391.7 (100%), 394.1 (68%)). In addition, the ratio of the relative intensity of the peaks corresponding to 4-Cl-L-Thr and 4,4-diCl-L-Thr for the reaction run in the presence of $[^{36}Cl]^-$ 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 covalent 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 obtained 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 addition. 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 increases 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

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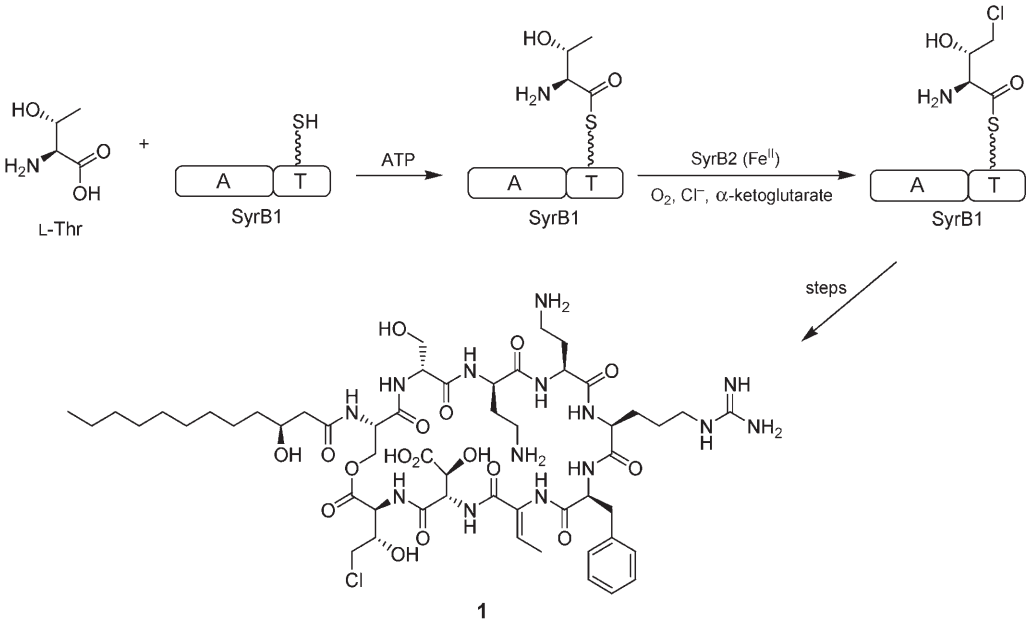
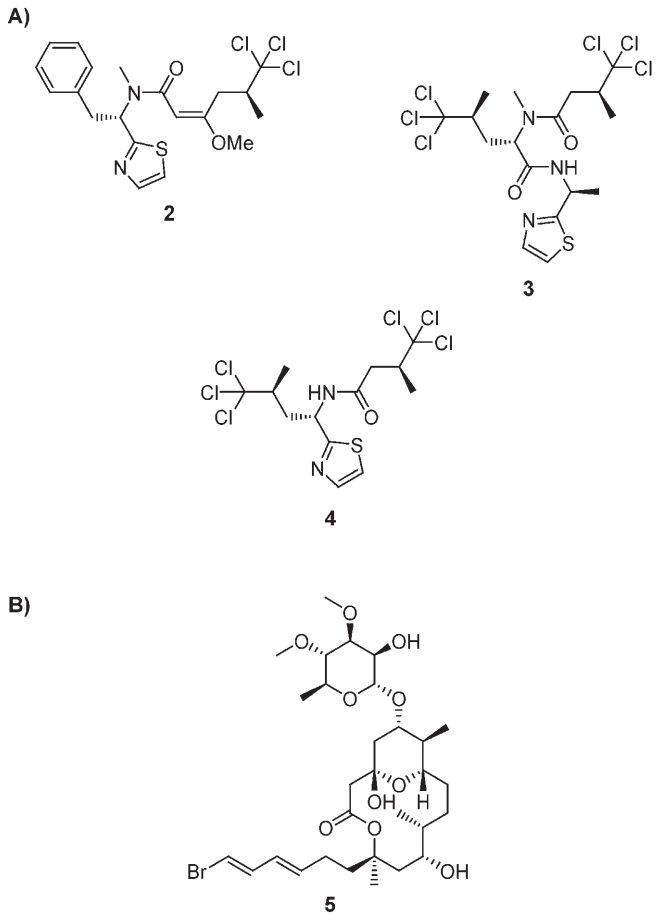


Scheme 1. Biosynthesis of 4-Cl-L-Thr, a key component of syringomycin E (**1**).



Scheme 2. Structures of chloride- and bromide-containing natural products. A) Aliphatic natural organohalogens containing three chlorines at a single carbon center: barbamide (**2**), dysidenin (**3**), and dysideathiazole (**4**). B) Structure of lyngbyaloside B (**5**).

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.[17] 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,[11] 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.[18] 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 mM bromide was present in the reaction mixture. By assuming free exchange of bromide with chloride in

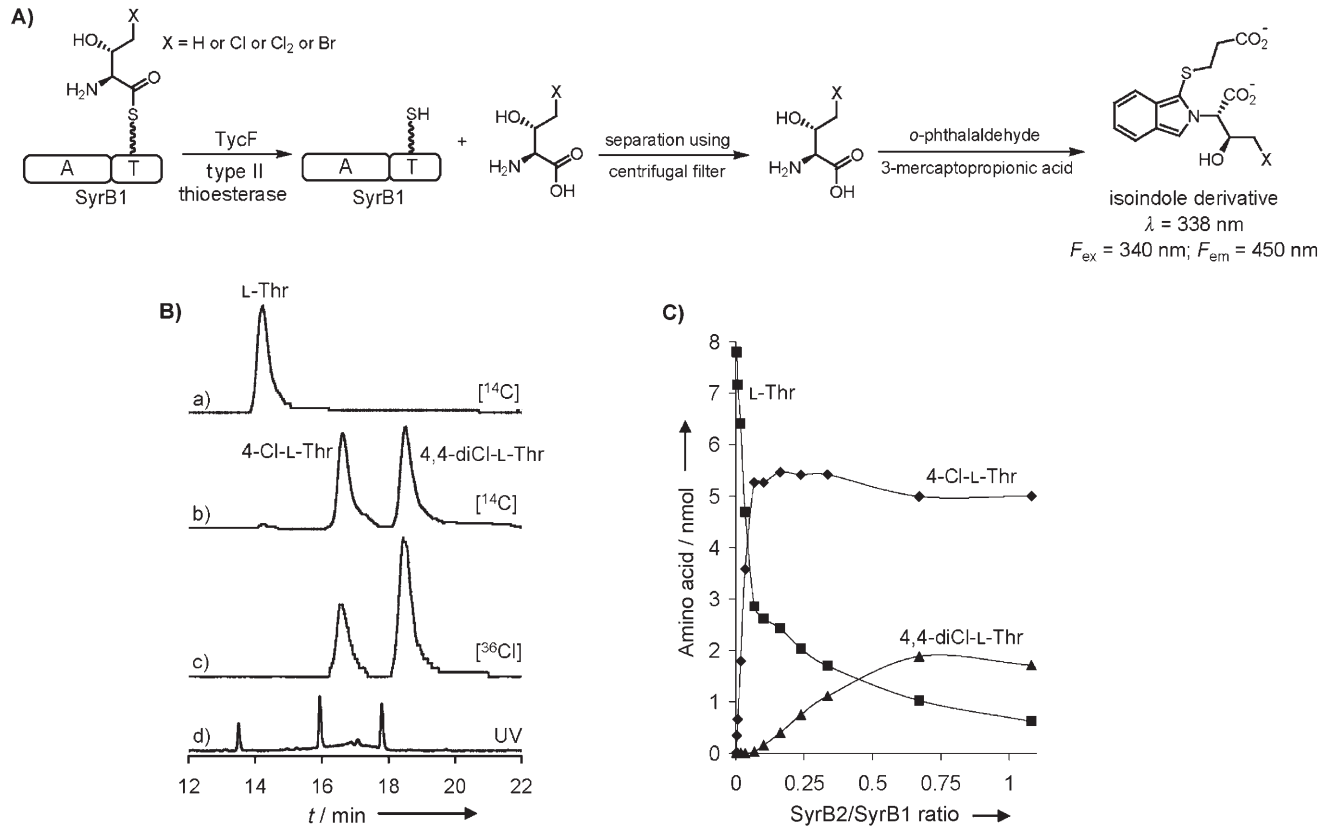


Figure 1. Biosynthesis of 4,4-diCl-L-Thr by the SyrB2 halogenase. A) Procedure for the preparation and derivatization of amino acids for HPLC analysis. B) Traces of hydrolyzed amino acid obtained after incubation of L-Thr-*S*-SyrB1 with SyrB2 (0.7:1 SyrB2/SyrB1). Radio-HPLC traces of a) the control reaction performed with L-[^{14}C]Thr-*S*-SyrB1 in the absence of SyrB2; b) the reaction performed with L-[^{14}C]Thr-*S*-SyrB1, chloride, O_2, α-ketoglutarate, and SyrB2; c) the reaction performed with L-Thr-*S*-SyrB1, O_2, α-ketoglutarate, and SyrB2 in presence of Na^{36}Cl. 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-*S*-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-*S*-SyrB1 in air-saturated buffer.

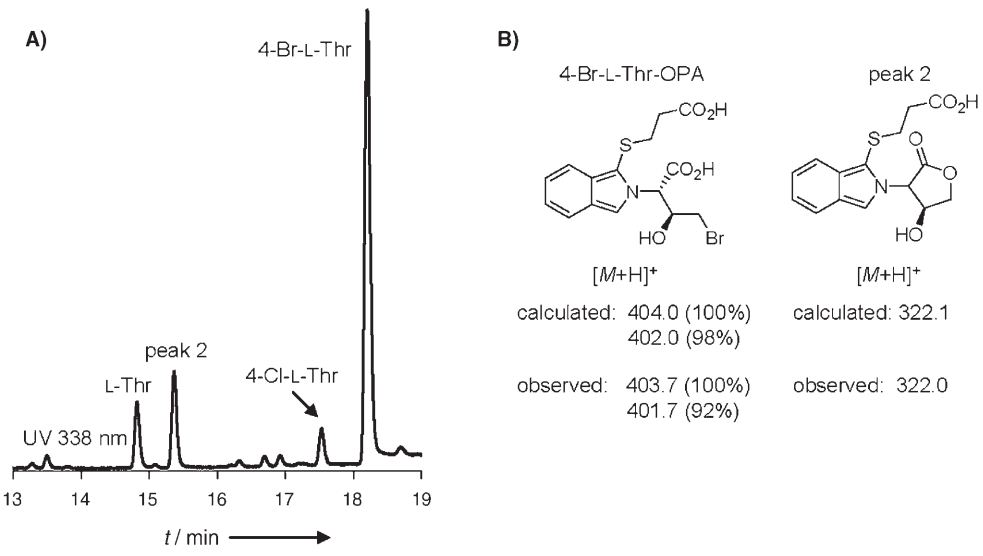


Figure 2. Biosynthesis of 4-Br-L-Thr by the SyrB2 halogenase. A) Trace of hydrolyzed amino acid obtained after incubation of 83 μM L-Thr-*S*-SyrB1 with 60 mM bromide, O_2, α-ketoglutarate, and 20 μM SyrB2. B) Mass spectrometric analysis of the isoindole derivative of 4-Br-L-Thr (together with its γ-lactone derivative) hydrolyzed from SyrB1.

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 2 B). 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 aminoacyl carboxylate. When the reaction was per-

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 γ-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 2 A). 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 $Cl^{\bullet}$ 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.[19] A solution of lithium hexamethyldisilazide (LHMDS; 1 M in THF, 2.3 mL, 2.3 mmol) was added to THF (3 mL) at −78 °C. A solution of Boc-Gly-O*t*Bu (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 NH_4Cl (7 mL), warmed to room temperature, and extracted with EtOAc (2×10 mL). The combined organic layers were washed with brine (1×10 mL), dried ($MgSO_4$), filtered, and concentrated in vacuo to give 0.5 g of a yellow oil. This oil was chromatographed on silica (10:1→2:1 hexanes/EtOAc) to remove the substantial amount of unreacted Boc-Gly-O*t*Bu, providing a yellow oil (58 mg) as the cleanest fraction. Further purification was difficult (C-18 preparatory HPLC, 40→55% MeCN in 0.1% trifluoroacetic acid over 20 min), but afforded Boc-4,4-diCl-Thr-O*t*Bu as a colorless film (8 mg, 2% overall yield). R_f = 0.14 (hexanes/Et_2O 5:1, eluted twice); ^{1}H NMR (200 MHz, $CDCl_3$): δ = 5.67 (d, J = 8.4 Hz, 1H), 5.16 (d, J = 9.4 Hz, 1H), 4.66 (dd, J = 1.8, 9.4 Hz, 1H), 4.32 (dd, J = 1.8, 8.4 Hz, 1H), 1.49 (s, 9H), 1.46 (s, 9H).

Boc-4,4-diCl-Thr-O*t*Bu (8 mg, 0.02 mmol) was dissolved in TFA (0.4 mL) and left at room temperature for 15 h. Volatiles were removed with a stream of nitrogen to give a tan film (6.9 mg, 100% yield). 4,4-DiCl-Thr was conveniently stored as its trifluoroacetic acid salt. ^{1}H NMR (500 MHz, D_2O): δ = 6.12 (d, J = 4.0 Hz, 1H), 4.42 (dd, J = 4.0, 5.5 Hz, 1H), 4.16 (d, J = 5.5 Hz, 1H).

Chemical synthesis of 4-Br-L-Thr: Cbz-3,4-epoxy-Abu-OBzl was prepared by the method of Webb and Matthews.[20] AcOH (61 μL, 1.1 mmol) followed by LiBr (49 mg, 0.57 mmol) were added to a solution of Cbz-3,4-epoxy-Abu-OBzl (121 mg, 0.35 mmol) in anhydrous THF (3.5 mL). The solution was stirred at room temperature for 2 h, then additional LiBr (0.1 g, 1 mmol) was added. After 21 h, the reaction mixture was diluted with hexanes/EtOAc (1:1, 10 mL), filtered through a short plug of silica, and concentrated in vacuo to give a yellow oil (0.3 g). The crude product was chromatographed on silica (hexanes/EtOAc 2:1→1:1) to provide Cbz-4-Br-Thr-OBzl (150 mg, 100% yield). R_f = 0.24 (hexanes/EtOAc 3:1); ^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.35 (br s, 10H), 5.84 (d, J = 9.0 Hz, 1H), 5.21 (s, 2H), 5.13 (s, 2H), 4.69 (d, J = 9.0 Hz, 1H), 4.42 (br s, 1H), 3.47 (m, 2H), 3.37 (m, 1H); ^{13}C NMR (125 MHz, $CDCl_3$): δ = 170.3, 157.1, 136.2, 135.3, 128.9, 128.8, 128.8, 128.6, 128.5, 128.4, 128.3, 72.0, 68.0, 67.7, 57.0, 34.8.

HBr (48% aqueous, 1 mL) was added to Cbz-4-Br-Thr-OBzl (9.7 mg, 23 μmol). The mixture was heated in an oil bath at 80 °C for 8 h, cooled to room temperature, washed with Et_2O (3×0.5 mL), frozen in liquid nitrogen, and lyophilized to afford a colorless oil (7 mg, 100% yield). 4-Br-L-Thr was conveniently stored as its hydrobromide salt. ^{1}H NMR (500 MHz, D_2O); δ = 4.32 (m, 1H), 4.15 (dd, J = 2.5, 4.0 Hz, 1H), 3.52 (dd, 2H, J = 2.5, 7.0 Hz).

Protein preparations and assays: The SyrB1 and SyrB2 proteins were purified and assayed as previously described.[10] The masses of the released amino acids from SyrB1 were also determined as previously described.[10] The only altered parameters were the SyrB2-L-Thr-*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,[10] prior to incubation with NaBr, O_2, α-ketoglutarate and SyrB2. Details of the concentrations that are different from previously described assays are in the figure legends.

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