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Enzymatic Regeneration of 3′-Phosphoadenosine-5′-Phosphosulfate Using Aryl Sulfotransferase for the Preparative Enzymatic Synthesis of Sulfated Carbohydrates

Michael D. Burkart, Masayuki Izumi, and Chi-Huey Wong*

Sulfotransferases comprise a family of enzymes that catalyze the transfer of a sulfo group (SO_3^-) from the cofactor 3′-phosphoadenosine-5′-phosphosulfate (PAPS, **1**) to an acceptor molecule. Sulfotransferases mediate the sulfation of different classes of substrates such as carbohydrates, proteins, and steroids for a variety of biological functions including signaling and modulation of receptor binding.[1, 2] Within the past three years many new sulfotransferases have been identified and cloned.[3] A facile means to produce large amounts of sulfated product and efficient sulfotransferase assays are essential for the biological study of these enzymes and their sulfated products.

The cofactor PAPS, the universal sulfate donor and source of sulfate for all sulfotransferases, is a highly expensive and unstable molecule that has been an obstacle to the large-scale production of enzymatically sulfated products.[4] Product inhibition by adenosine 3′,5′-diphosphate (PAP, **2**) has also been a limiting factor to large-scale applications.[5, 6] We reported the first enzymatic regeneration of PAPS based on a multienzyme system coupled with *Rhizobium* Nod factor sulfotransferase (NodST) for the enzymatic synthesis of *N*,*N*′-diacetylchitobiose 6-sulfate.[6] While application of the cloned enzymes used in this recycling system serve as a facile and efficient means to synthesize PAPS on a large scale, we have developed here a simpler, one-enzyme regeneration system using a recombinant rat liver aryl sulfotransferase IV (AST IV; Scheme 1). This enzyme, when coupled to a sulfotransferase of choice, transfers a sulfo group (SO_3^-) from *p*-nitrophenyl sulfate (**3**) to PAP.[7] This system averts product inhibition by PAP while regenerating PAPS in situ and can be monitored quantitatively by measurement of the absorbance of released *p*-nitrophenol (**4**) at 400 nm. Additionally, this recycling system can be modified to serve as a continuous spectrophotometric assay for the activity of any sulfotransferase enzyme. The most common assay of sulfotransferase activity involves monitoring the transfer of radioisotopic sulfate from [^{35}S]PAPS to the product by thin-layer or paper chromatography using end-point methods.[8] These assays are expensive and often imprecise.[9] Demonstrated here is the use of this one-enzyme PAPS regeneration system for the synthesis of *N*,*N*′,*N*″-triacetylchitotriose 6-sulfate (**5**) and of a continuous, colorimetric, coupled-enzyme assay of NodST to determine the substrate specificity of this enzyme.

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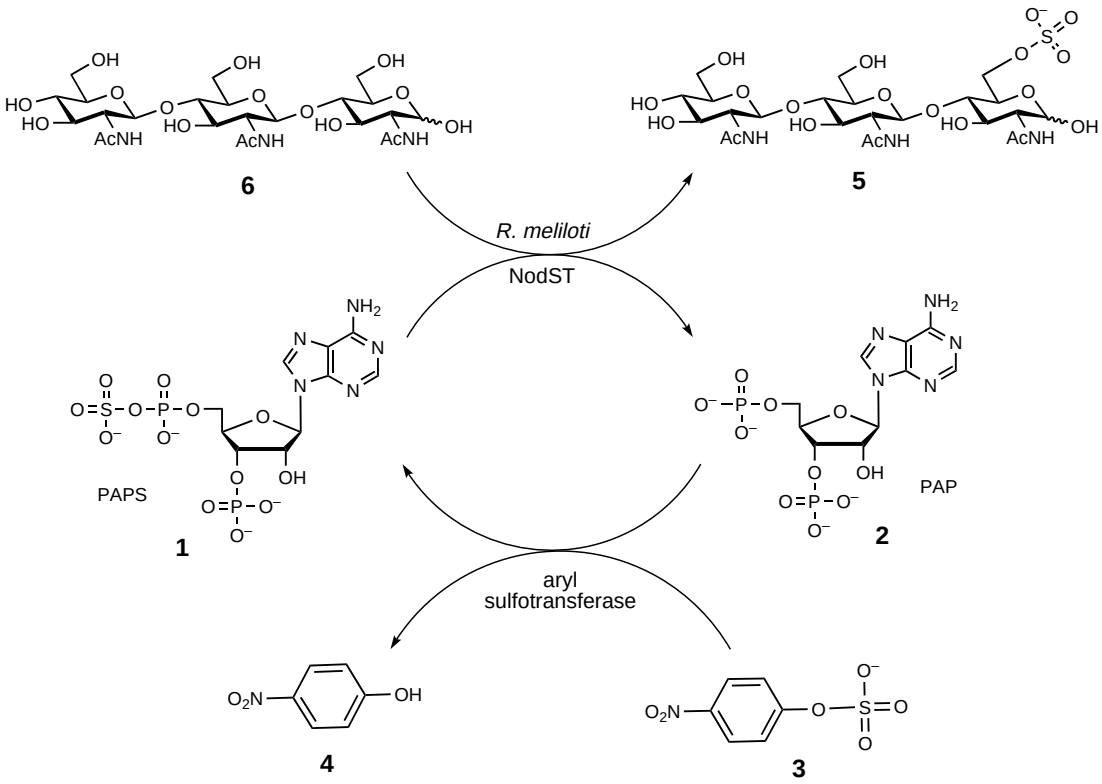

Scheme 1. Regeneration of PAPS with recombinant aryl sulfotransferase and *p*-nitrophenyl sulfate.

The enzyme AST IV exists in two forms with different oxidation states[10, 11] that can be easily resolved, and the isolated physiologically relevant form has been utilized to assay picomole quantities of PAPS and PAP.[12] Since the bacterial expression of rat T IV has been demonstrated,[13, 14] we cloned AST IV from a rat liver cDNA library and overexpressed the enzyme in *Escherichia coli*. To obtain the pure β form of the enzyme, in which PAP is not bound in the active site, conversion of the mixed enzyme forms was simply carried out during purification through nickel-agarose chromatography. The enzyme-bound nickel resin was incubated with alkaline phosphatase and β-mercaptoethanol (BME) prior to elution. The purified enzyme was then immediately available for PAPS regeneration. Capillary zone electrophoresis (CZE) was employed to distinguish between the ability of AST IV to form PAPS from PAP and *p*-nitrophenyl sulfate rather than effect mere hydrolysis of *p*-nitrophenyl sulfate, and it verified the production of PAPS from PAP.

N,*N*′,*N*″-Triacetylchitotriose 6-sulfate (207 mg) was synthesized in 58% yield from commerically available NodST and the β form of AST IV with 0.05 equivalents of PAP and 2.0 equivalents of *p*-nitrophenol sulfate. The structure was confirmed by electrospray ionization (ESI) mass spectrometry, HPLC, and NMR spectroscopy. The same procedure was used in the synthesis of *N*,*N*′,*N*″,*N*‴-tetraacetylchitotetraose 6-sulfate (150 mg, 95% yield).

The continuous assay for kinetic analysis of sulfotransferases resembles the large-scale methodology with the exception that PAPS is added to the reaction in place of PAP, since the purpose of the assay is the continuous measurement of PAP production. The assay utilizes the β form of AST IV described above. To demonstrate the application of this assay to the determination of enzymatic kinetic parameters, *N*,*N*′,*N*″-triacetylchitotriose at concentrations of 10, 25, 50, 100, 250, and 500 μM was used as substrate. The data were fitted to the Michaelis–Menten equation with the nonlinear, least-squares program LineWeaver[15] to yield $K_m = 103$ μM, $k_{cat} = 27.5\ min^{-1}$ (Figure 1). Specificity experiments for the acceptor sugar were also conducted to probe the substrate specificity of NodST with this assay. Table 1 contains the data for various *N*-acetylchitoses, *N*-acetyllactosamine (LacNAc), and an *N*-acetyllactosamine dimer (Gal(β1-4)GlcNAc(β1-

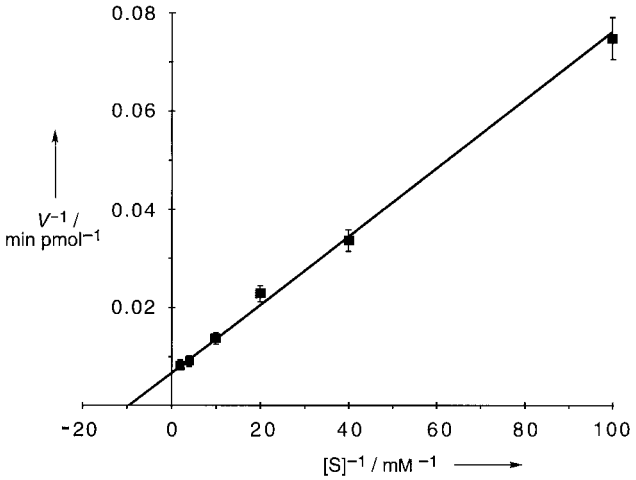

Figure 1. Lineweaver–Burke plot of kinetic data for the treatment of *N*,*N*′,*N*″-*triacetylchitotriose with NodST.* $K_m = 20.4 \pm 3.6$ μM, $k_{cat} = 19.7 \pm 0.8\ min^{-1}$.

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Table 1. Acceptor specificity for NodST.[a]

Acceptor substrate	K_M [μM][b]	k_{cat} [min^{-1}]
GlcNAc(β1-4)GlcNac	243 ± 40	23.6 ± 1.7
GlcNAc(β1-4)GlcNac(β1-4)GlcNAc	103 ± 12	27.5 ± 2.9
GlcNAc(β1-4)GlcNac(β1-4)GlcNAc(β1-4)GlcNAc	20.4 ± 3.6	19.7 ± 0.8
GlcNAc(β1-4)GlcNac(β1-4)GlcNAc(β1-4)GlcNAc(β1-4)GlcNAc	98.1 ± 12.4	25.9 ± 1.2
GlcNAc(β1-4)GlcNAc(β1-4)GlcNAc(β1-4)GlcNAc(β1-4)GlcNAc(β1-4)GlcNAc	45.7 ± 6.0	22.7 ± 1.0
Gal(β1-4)GlcNAc	> 5000	8.0 ± 1.3
Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)GlcNAc	87.0 ± 6.3	3.4 ± 0.1

[a] The acceptor sugar concentrations were chosen around their respective K_m values. The PAPS concentration was held constant at 20 μM. Kinetic constants were derived from the nonlinear, least-squares fit of the Michaelis–Menten equation with the LineWeaver program. [b] Apparent kinetic values.

3)Gal(β1-4)GlcNAc).[16] Not surprisingly, *N*,*N*′,*N*″,*N*‴-tetraacetylchitotetraose proved to be the best substrate (k_{cat} = 19.7 min^{-1}, K_m = 20.4 μM), as it is the most similar to the natural substrate.[17] Of particular interest is the 60-fold increase in acceptor specificity from LacNAc to the LacNAc dimer.

In summary, this new enzymatic regeneration of PAPS appears to be more practical and convenient than previously reported methods for the synthesis of carbohydrate sulfates and kinetic analysis of sulfotransferases. This regeneration system should be generally applicable to other sulfotransferases.

Experimental Section

Capillary zone electrophoresis was performed on a BioRad 3000 apparatus with a coated capillary (50 cm, 50 μm inner diameter) in 0.23 M borate in running buffer (pH 7.8). The sample was injected at a pressure of 138 kPa s^{-1}, and electrophoresis was run with 20.0 kV (− to +) at 20 °C with detection at 260 nm. PAPS was detected at 5.53 min, and PAP at 5.98 min.

The AST IV gene was obtained by polymerase chain reaction as described[11] from a cDNA library template (provided by W. E. Balch, The Scripps Research Institute). The clone, containing an engineered *Nde*I site preceding the 5′ coding region and a *Bam*HI site following the 3′ stop codon, was digested with *Bam*HI and *Nde*I and ligated with a similarly digested PET19b (Novagen, Madison, WI). The ligated plasmid was transformed into *E. coli* SURE (Stratagene, La Jolla, CA) for amplification. *E. coli* BL21(DE3) (Novagen) was transformed with the amplified plasmid, and a single colony was chosen to innoculate 100 mL of Luria–Bertani (LB) broth containing 50 μg mL^{-1} of ampicillin. After overnight growth at 37 °C, the culture was distributed into four 1-L volumes of LB with ampicillin and grown at 37 °C to an optical density at 600 nm (OD_{600}) of 1.0. The cultures were induced with 50 μM diisopropyl-β-D-thiogalactopyranoside (IPTG), grown at 25 °C for 20 h, and centrifuged. The cell pellet was resuspended in 100 mM tris(hydroxymethyl)aminomethane (Tris, pH 7.6) with 5 mM 2-mercaptoethanol and disrupted by a French press. The supernatant was loaded onto a nickel–agarose column and washed with a solution of 100 mM Tris (pH 7.6), 5 mM 2-mercaptoethanol, 300 mM NaCl, 5% glycerol, and 20 mM imidazole followed by equilibration with 100 mM Tris (pH 9.0), 50 mM NaCl, and 5 mM BME. Alkaline phosphatase (500 U) was added, and the resin was incubated at 20 °C for 15 h. After the resin was washed as described above, the β form of AST IV was eluted from the column with 100 mM Tris (pH 7.6) and 250 mM imidazole. Dialysis into 100 mM Tris (pH 7.6), concentration, and dilution to 50% glycerol yielded 63 U of enzyme (15.8 U L^{-1}) in 14 mL (4.5 U mL^{-1}) of glycerol stock. One unit (U) is defined as the convertion of 1 μmol of PAP into PAPS per minute with *p*-nitrophenyl sulfate at pH 7.0, 25 °C.

5: *N*,*N*′,*N*″-triacetylchitotriose (314 mg, 500 μmol) in a solution (100 mL) of bis[tris(hydroxymethyl)methylaminopropane] hydrochloride (bis-Tris-propane-HCl (100 mM, pH 7.0) with 5 mM dithiothreitol and 10 mM *p*-nitrophenyl sulfate, 11 mg of PAP (25 μmol), 8.4 U of NodST (Calbiochem, La Jolla, CA), and 18 U of β-AST IV were incubated at room temperature. Formation of *N*,*N*′,*N*″-triacetylchitotriose 6-sulfate was monitored by TLC (*n*-propanol/30% NH_4OH/H_2O 7/1/2) and liberation of *p*-nitrophenol by the absorbance at 400 nm. After three days, the solution was passed through a column of LiChroPrep 18 (water). The void fraction was absorbed to a BioRad AG1-X8 column (HCO_2^- form) and eluted with a linear gradient (0 → 0.1 M) of ammonium benzenesulfonate. Fractions containing *N*,*N*′,*N*″-triacetylchitotriose 6-sulfate were concentrated and purified by size-exclusion chromatography (BioGel P-2, water) to yield 207 mg of **5** (290 μmol, 58%). Positive-ion ESI MS: *m*/*z*: *N*,*N*′,*N*″-triacetychitotriose 6-sulfate 730 [M+Na^+], the degradation product *N*,*N*′,*N*″-triacetylchitotriose 628 [M+H^+], 1-deoxy-*N*,*N*′-diacetylchitobiose 407 [M^+], and 1-deoxy-*N*-acetylglucosamine 204 [M^+] (for comparison, see the original Nod signal characterization[18]); HPLC (Spherisorb S5 SAX column (Waters 4.6 × 250 mm) with a linear gradient of 0.2 → 1.0 M NaCl over 5 min in 10 mM KH_2PO_4 (pH 3.5), flow rate 1.0 mL min^{-1}, and detection at 220 nm): Two peaks (4.12, 4.37 min) correspond to the α and β anomer of the reducing end; ^{1}H NMR (500 MHz, D_2O): δ = 4.25 (br, 0.4 H, *J* = 10.3 Hz, H6aβ), 4.20 (dd, 0.6 H, *J* = 1.8, 11.0 Hz, H6aα), 4.16 (dd, 0.6 H, *J* = 3.7, 11.0 Hz, H6bα), 4.12 (dd, 0.4 H, *J* = 3.7, 9.6 Hz, H6bβ); ^{13}C-NMR (125 MHz, D_2O): δ = 67.04 (C6α), 66.97 (C6β).

Assay of NodST: 100 mM bis-tris-propane-HCl (pH 7.0), 5 mM 2-BME, 20 μM PAPS, 2 mM *p*-nitrophenyl sulfate, 3.0 mU of NodST, 6.4 mU of β-AST IV, and substrate are combined to a total volume of 500 μL. The assay solution is incubated for 30 min at 20 °C prior to addition of substrate because commercial PAPS contains significant amounts of PAP. The evolution of *p*-nitrophenol is monitored at 400 nm for 10 min.

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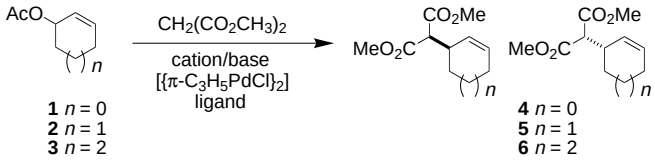
Proline-Based P,N Ligands in Palladium-Catalyzed Asymmetric π-Allyl Additions**

Scott R. Gilbertson* and Dejian Xie

The development of new ligands for use in asymmetric catalysis has undergone incredible growth in the last ten years. One of the reactions that has been investigated extensively is the palladium-catalyzed addition of nucleophiles to allyl acetates.[1, 2] One of the first substrates to yield to catalysis with high selectivity was 1,3-diphenylprop-2-enyl acetate.[3–11] There are now a number of catalysts that have been developed that will catalyze the asymmetric addition of malonate to this substrate in greater than 90 % enantiomeric excess. A type of substrate that has been significantly more difficult to achieve high selectivity with has been the cyclic allyl acetates **1** – **3** (Scheme 1).[12–15] To the best of our knowledge there are currently only two systems that give greater than 95 % *ee* with these types of substrates.[16–22] Herein we report the synthesis of new phosphanyldihydrooxazole ligands that can be used to catalyze this substitution in high enantiomeric excess. The results obtained with these ligands are comparable to the most selective catalysts for the reaction of this substrate. Additionally, these ligands are readily accessible from common amino acids and are proving useful in the catalysis of a number of asymmetric transformations.



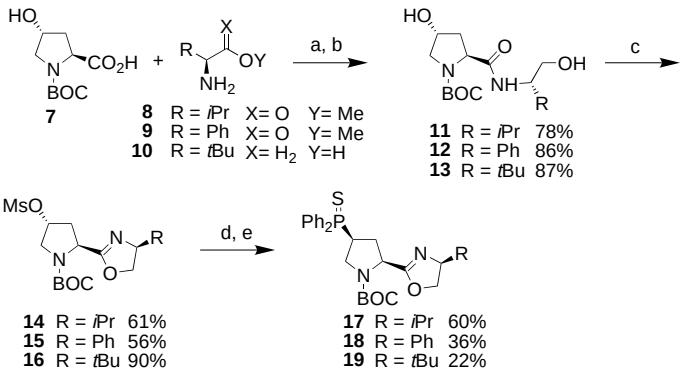
1 *n* = 0
2 *n* = 1
3 *n* = 2

4 *n* = 0
5 *n* = 1
6 *n* = 2

Scheme 1. Asymmetric addition of dimethylmalonate to cyclic allyl acetates **1** – **3**.

The design of this system was founded on the observation that ligands based on proline often result in reasonably high stereodifferenation. There have been a number of examples of proline-based bisphosphane systems that have resulted in selective catalysis.[23–27] For this reason a series of proline-based phosphanyldihydrooxazole ligands was synthesized.

The synthesis of this class of ligand begins with commercially available *tert*-butoxycarbonyl (BOC)-protected *trans* 4-hydroxy-L-proline (**7**) which could be coupled to either an amino acid ester or an amino alcohol (Scheme 2). The dihydrooxazole was formed through reduction of the ester



Scheme 2. Synthesis of the dihydrooxazole ligands **17** – **19**. EDC/HOBt, RT, CH_2Cl_2; b) when X = O, $LiBH_4$, THF, 0 °C to RT; c) $MeSO_2Cl$, Et_3N, CH_2Cl_2, RT; d) $Ph_2P^-Na^+$, THF, – 78 °C to RT; e) S_8 or $Na_2S_2O_2$, CH_3OH/H_2O, 45 °C. EDC = 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, HOBt = 1-hydroxy-1*H*-benzotriazole. Ms = mesyl.

to an alcohol and formation of a bis-mesylate by reaction with methanesulfonyl chloride. The primary mesylate then underwent cyclization to form the dihydrooxazole ring (**14** – **16**). After this reaction, the secondary mesylate was subjected to substitution by sodium diphenylphosphide. Following substitution the phosphane is protected as the phosphane sulfide. The ligands are purified as the phosphane sulfides and then converted to phosphanes (**20** – **25**) by reduction with Raney

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