Molecular Basis of Substrate Promiscuity for the SAM-Dependent O-Methyltransferase NcsB1, Involved in the Biosynthesis of the Enediyne Antitumor Antibiotic Neocarzinostatin†

Heather A. Cooke,§ Elizabeth L. Guenther,§ Yinggang Luo,‖ Ben Shen,‖,1 and Steven D. Bruner*§

§Department of Chemistry, Merkert Chemistry Center, Boston College, Chestnut Hill, Massachusetts 02467, †Division of Pharmaceutical Sciences and ‡Department of Chemistry, University of Wisconsin, Madison, Wisconsin 53705

Received July 22, 2009; Revised Manuscript Received August 20, 2009

ABSTRACT: The small molecule component of chromoprotein enediyne antitumor antibiotics is biosynthesized through a convergent route, incorporating amino acid, polyketide, and carbohydrate building blocks around a central enediyne hydrocarbon core. The naphthoic acid moiety of the enediyne neocarzinostatin plays key roles in the biological activity of the natural product by interacting with both the carrier protein and duplex DNA at the site of action. We have previously described the in vitro characterization of an S-adenosylmethionine-dependent O-methyltransferase (NcsB1) in the neocarzinostatin biosynthetic pathway [Luo, Y., Lin, S., Zhang, J., Cooke, H. A., Bruner, S. D., and Shen, B. (2008) J. Biol. Chem. 283, 14694–14702]. Here we provide a structural basis for NcsB1 activity, illustrating that the enzyme shares an overall architecture with a large family of S-adenosylmethionine-dependent proteins. In addition, NcsB1 represents the first enzyme to be structurally characterized in the biosynthetic pathway of neocarzinostatin. By co-crystallizing the enzyme with various combinations of the cofactor and substrate analogues, details of the active site structure have been established. Changes in subdomain orientation were observed via comparison of structures in the presence and absence of substrate, suggesting that reorientation of the enzyme is involved in binding of the substrate. In addition, residues important for substrate discrimination were predicted and probed through site-directed mutagenesis and in vitro biochemical characterization.

Enediyne antitumor antibiotics are structurally complex natural products possessing remarkable cytotoxicity (1). Members of the nine-member subfamily of enediynes are composed of a small molecule chromophore and an apoprotein, which sequesters and stabilizes the reactive enediyne and aids in delivery to target cells, leading to single- and double-stranded DNA cleavage (2). Biosynthetic gene clusters of multiple enediyne from actinomycetes have recently been sequenced and annotated, allowing detailed investigation into their complex biosynthesis (3–8). The pathways utilize diverse enzymology to construct the natural products, including the use of polyketide and nonribosomal peptide machinery. Neocarzinostatin (NCS,1 1) is an archetypal example of the nine-membered enediyne, the studies of which have contributed to the general understanding of enediyne biosynthesis and mode of action (4, 9–12). The naphthoate moiety of NCS functions by both binding to the apoprotein and intercalating target DNA, thereby positioning the enediyne in the minor groove (9, 10, 13–15). Alterations of the peripheral moieties of NCS surrounding the enediyne core...
Rsderved small molecule MTase fold (26) revealed a homodimer with each monomer exhibiting the consequence identity (25). The enzyme involved in the biosynthesis of the aromatic polyketide β-structure with a glycine-rich region within the SAM binding pocket. Despite a low degree of overall sequence homology among family members, structurally characterized SAM-dependent O-MTases, including DnrK and RebM from rebeccamycin biosynthesis (32).

Here we report the structural basis for NcsB1 activity in a variety of cocomplexes with SAM or 5-adenosyl-l-homocysteine (SAH) with or without 5-methyl-2-hydroxynaphthoic acid (2), product (4), or 1,4-dihydroxy-2-naphthoic acid (6), an alternate substrate. The structures reveal that NcsB1 shares an overall architecture common to the large MTase family. The active site binding pocket is able to accommodate the natural substrate and structurally diverse analogues, allowing efficient methylation with distinct regiospecificity. The specificity determinants of the naphthoate binding pocket were probed using site-directed mutagenesis and alternate substrates. On the basis of the results, residues that affect the substrate specificity of the enzyme were identified.

MATERIALS AND METHODS

Protein Expression and Purification. NcsB1 was overproduced as an N-terminal His$_6$-tagged fusion protein using expression plasmid pBS5039 in Escherichia coli BL21(DE3) cells as reported previously (21). Cells were grown in 1 L of Luria-Bertani medium at 37 °C and 150 rpm to an OD$_{600}$ of 0.5–0.8. Overexpression was induced with 50 μM isopropyl β-D-thiogalactopyranoside at 18 °C for 16 h. Cells were collected by centrifugation (20 min at 2000×g), resuspended in 25 mL of 20 mM Tris-HCl (pH 7.5) and 500 mM NaCl, and flash-frozen at −78 °C. Cell pellets were thawed and lysed via two passes through a French press cell disruptor at 1000 psi. The lysate was then clarified by centrifugation (20 min at 10000g), and the protein was purified batchwise using Ni-NTA resin (Qiagen, Valencia, CA). NcsB1 used in biochemical assays was dialyzed into 20 mM Tris-HCl (pH 7.5), 50 mM NaCl, and 1 mM β-mercaptoethanol, then concentrated using an Amicon Ultra-4 concentrator (10 kDa molecular mass cutoff, GE Healthcare), and frozen with 40% supplemented glycerol at −25 °C.
For crystallography, the Ni-NTA-purified protein was dialyzed into 20 mM Tris-HCl (pH 7.5), 100 mM NaCl, 2 mM CaCl₂, and 1 mM β-mercaptoethanol and concentrated to ~1 mL. The His₆ tag was cleaved by incubation with protase Factor Xa for 36 h at 4°C (monitored by SDS–PAGE). The protein solution was then diluted to 5 mL and purified on a HiTrap-Q ion exchange column [20 mM Tris-HCl (pH 7.5), 100 mM NaCl, 2 mM CaCl₂,a n d β-mercaptoethanol, and 10% glycerol]. The purified protein was concentrated to ∼10 mg/mL for crystallography (a total yield of 654 mg). Mature crystals were transferred to a cryoprotectant solution (4.0 M sodium formate and 15% glycerol) and soaked briefly before being flash-frozen in liquid nitrogen. X-ray diffraction data were collected on beamline X25 at the National Synchrotron Light Source at Brookhaven National Laboratories with an ADSC Q315 CCD X-ray detector. Diffraction intensities were measured with a 15° oscillation width and a pixel size of 0.2° and 0.145° pixel size, respectively.

**Table 1: Data Collection and Refinement Statistics**

<table>
<thead>
<tr>
<th></th>
<th>SAH</th>
<th>SAM and 2</th>
<th>SAH and 4</th>
<th>SAH and 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>space group</td>
<td>C222₁</td>
<td>P6₃</td>
<td>P6₃</td>
<td>P6₃</td>
</tr>
<tr>
<td>unit cell parameters (Å)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>a = 91.3</td>
<td>a = b = 109.1</td>
<td>a = b = 108.4</td>
<td>a = b = 108.0</td>
<td></td>
</tr>
<tr>
<td>b = 161.6</td>
<td>c = 206.9</td>
<td>c = 210.3</td>
<td>c = 211.9</td>
<td></td>
</tr>
<tr>
<td>c = 98.9</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Data Collection**

- resolution (Å): 25.0–2.08 (2.15–2.08) 30.0–2.69 (2.79–2.69) 50.0–3.0 (3.1–3.0)
- wavelength (Å): 1 1 1
- Rmerge: 0.052 (0.349) 0.077 (0.441) 0.094 (0.49) 0.076 (0.523)
- I/σ(I): 49.6 (6.4) 24.0 (7.0) 29.3 (4.5)
- redundancy*: 7.8 (7.4) 17.3 (17.8) 12.6 (12.8)
- no. of reflections (total/test): 41442/2071 41357/2064 37165/3704 26923/2698
- completeness (%): 99.6 (99.6) 99.8 (100.0) 98.5 (100.0) 99.8 (100.0)
- Rwork: 0.240 0.206 0.212 0.217
- Rfree: 0.272 0.227 0.247 0.245
- no. of amino acids (chains A/B): 325/303 325/325 328/328 328/328
- no. of water molecules: 305 249 163 95
- no. of ligands (cofactor/substrate/glycerol): 52/−6/30 54/30/12 52/34/12 50/30/12
- Ramachandran plot by PROCHECK (%):
  - overall (highest-resolution shell):
    - core region: 90.6 93.2 91.7 88.8
    - allowed region: 9.0 6.2 7.9 10.4
    - generously allowed: 0.2 0.5 0.2 0.7
    - disallowed: 0.2 0.0 0.2 0.0

**Refinement**

- root mean square differences (Å):
  - bonds: 0.006 0.006 0.006 0.0013
  - angles (deg): 1.2 1.3 1.2 1.7
- PDB entry: 3I64 3I64 3I64 3I64

*Overall (highest-resolution shell).
indexed, integrated, and scaled using HKL2000 (33) as summarized in Table 1. The crystals belonged to space group P65 except for the NesB1–SAH cocomplex, which belonged to space group C2221.

**Structure Determination.** An initial solution for the NesB1–SAM–2 ternary complex was obtained using the molecular replacement program Phaser (34), part of the CCP4 suite (35). A polyalanine dimer model of DnrK from S. peucetius (PDB entry 1TW2) truncated to residues 15–350 was used as the search model. Manual building of the NesB1 model was performed using COOT (36), and the structure was refined using CNS (37, 38). This solution was used to solve phases in subsequent data sets in the P65 space group using molecular replacement. For the NesB1–SAH cocomplex (C2221 space group), a partial solution (chain A and the N-terminus of chain B) was found using Phaser. The remaining C-terminus of chain B was placed into the model using MOLREP (39). All final structures were subjected to multiple rounds of building and refinement until the R values converged. Noncrystallographic restraints were used for all P65 space group structures until the final stages of refinement. Ligands were fit into the electron density maps, and the PRODRG server (40) was used for generating topology and parameter files; SAM and SAH coordinates were obtained from the HICup server (41) and were subsequently altered to fit electron density. PyMOL (DeLano Scientific, San Carlos, CA) was used to generate graphic images. rmsd values were calculated via structural comparison using TopMatch (42, 43).

The NesB1–SAH structure (C2221 space group) was determined at a resolution of 2.08 Å and refined to Rwork and Rfree values of 24.0 and 27.2%, respectively (Table 1). The asymmetric unit consisted of two monomers. For chain A, there was sufficient electron density to build in residues 5–330 and the bound SAH molecule. For chain B, the final model consisted of residues 9–150, 159–274, 282–297, and 302–330, as insufficient electron density was available to build the 29 remaining residues. Electron density for the SAH molecule was well-defined, albeit with higher density was available to build the 29 remaining residues. Electron density to build in residues 5–330 and methylated product

RESULTS AND DISCUSSION

**Crystallography and X-ray Structure Determination.** The 34.5 kDa/332-residue NesB1 was cocry stallized with various combinations of SAH, SAM, deshydroxy substrate analogue 2, and methylated product 4. Analogue 2 was used in crystallization experiments because of the oxidative instability of the substrate (3) over extended time periods. Two crystal forms were generated depending on the nature of the bound small molecules. When cocry stallized with a substrate analogue/product and SAH or SAM, the cocomplex crystallized in the P65 space group. In the absence of naphthoic acid, distinct crystals belonging to the C2221 space group formed. The NesB1–SAM–2 ternary complex was the initial structure determined using molecular replacement with a polyalanine dimer model of DnrK (44% identical) as the search model. This structure was then used as a search model for the alternate cocomplexes. Solution of the NesB1–SAH complex (C2221 space group) by molecular replacement was not straightforward, suggesting an alternate overall structure. The final structural model was obtained by performing rotation and translation searches separately on the N- and C-terminal domains. While a 0.6 Å rmsdca was observed among the P65 structures, the NesB1–SAH structure differed significantly from the others by an rmsdca of 1.8 Å. In addition, the two subunits of the NesB1–SAH structure were quite distinct, with an rmsdca of 2.8 Å between the homodimer chains.

**Overview of the Structures.** The resulting dimer models of NesB1 display a high degree of structural similarity with DnrK (rmsdca = 1.8 Å). In the initial NesB1–SAM–2 structure, and others in the P65 space group, the two monomeric subunits exhibit a high degree of symmetry (Figure 2A). Each subunit of the NesB1 dimer is made up of three domains totaling 19 α-helices and eight β-sheets (Figure 2B). The N-terminal domain is largely α-helical, with just two β-strands (β1 and β2), and constitutes most of the residues involved in the homodimer interface (~5300 Å2 total surface). Of note, α5 (residues 52–65) has significant interactions with α18 (residues 284–292) from the C-terminal domain of the paired dimer. The dimer interface consists of primarily hydrophobic residues, and a dimeric biological unit was supported by size-exclusion chromatography (Figure S1 of the Supporting Information). A middle domain acts as a hingelike region between the two larger terminal domains and consists of α-helices 9–11. The C-terminal domain exhibits a Rossmann-like fold (β3–9 flanked by α12–19), which is conserved in structural homologues and makes up the majority of the SAM binding site (24). On the basis of the structural alignment using the DALI server, NesB1 shares the highest
degree of structural similarity with SAM-dependent enzymes that act on small molecules (Figure 3 and Table S2) (44). Homologues include DnrK (an O-MTase) and RdmB (a hydroxylase), iso-
flavone-O-MTase from alfalfa (45), a putative O-MTase from
*Nostoc punctiforme*, a phenazine-specific N-MTase from *Pseu-
domonas aeruginosa* (46), and caffeic acid 3-O-MTase from
alfalfa (47). Among the structures, an indicative glycine-rich
region of the SAM binding pocket is conserved. These six
structural homologues demonstrate the ability of the conserved
scaffold to catalyze a variety of transformations.

In the NcsB1–SAH cocomplex structure, the C-terminal
domain of chain B is rotated by ~20° compared to that in the
NcsB1–SAH–2 structure, displacing α13 by 6.8 Å and β8/β9 by
9.4 Å (Figure 2C). This movement results in a more open
conformation of the active site, possibly functioning to allow
the entrance of substrate. A similar domain displacement was
seen in the DnrK ternary complex where two crystals with
different space groups were also observed (26). Both DnrK
crystals were bound to SAH and product, but the B factors were
higher in one of the two subunits, suggesting a lower occupancy
of the ligands. One of the crystal structures exhibited a significant
difference between the two monomers, with an rmsd_{ca} of 1.3 Å.
Likewise, the RdmB structures exhibit a domain movement upon
substrate binding, though in this cocomplex structure, both
domains were displaced (27). The NcsB1–SAH cocomplex
structure here exhibits a larger difference, likely due to a
complete lack of substrate analogue or product under the
crystallization conditions. In addition, SAH bound in the open
subunit (chain B) has higher relative B factors. Several regions in
chain B exhibited weak or no electron density, possibly indicating
areas of flexibility on which the domain can twist. These hinge
regions include part of α1 (residues 150–159), the loop region
between β7 and α18 (residues 274–282), and the loop between
α18 and α19 (residues 297–301) (Figure 2B).

*SAM/SAH Binding Pocket.* The SAM/SAH binding
pocket is located at the C-terminus of the β-strands comprising part
of the Rossmann-like fold. Electron density for SAM or SAH is
clearly defined in all four cocomplex structures, and the SAM
binding interactions are largely conserved among small molecule
MTases. The adenine ring is involved in a hydrogen bonding
interaction with Ser227, a π interaction with Phe228, and the
binding pocket is lined with additional aromatic and/or hydro-
phobic residues, including Trp133, Trp248, Phe229, and Leu201
(Figure 4A). The ribosyl moiety is anchored by two hydrogen
bonds between Asp200 and Ser143, and the homocysteine portion
of SAM forms hydrogen bonding interactions with the side chains
of Asp175, Ser242, and His153 and the backbone carbonyl of
Gly177. The LDGXGXGX motif indicative of SAM-utilizing
proteins is found in NcsB1 as VDVGGGSG and is located
between β3 and α14. We previously reported that NcsB1 alkylates
substrate 3 with a variety of SAM analogues, including S-ethyl and
S-n-propyl, to the corresponding 7-alkyl ether with reasonable
efficiency (21). The side chain closest to the methyl group on SAM
is the sterically small Ala243 (3.5 Å distance). On the basis of the
large size and flexibility of the substrate binding pocket, it is not
surprising that NcsB1 could accommodate larger SAM analogues.

*Naphthoic Acid Binding Pocket.* The naphthoic acid bind-
ing site is located at the juncture of helices from all three
subdomains, including α7, α10, α11, α16, and α18, and
the pocket is lined primarily with hydrophobic/aromatic residues
(Figure 4A). Three methionine side chain thioethers (Met150,
-286, and -290) are present on either side of the naphthoate ring
forming van der Waals interactions with the substrate. The
carboxylate and 2-hydroxyl groups of the substrate interact with
Arg11 directly and with Asp157 through an ordered water. This
interaction represents the only hydrogen bonds between the
enzyme and substrate and appears to anchor the naphthoic acid
into the active site adjacent to the bound SAM. To probe the
importance of Arg11 in enzyme activity, we used site-directed
mutagenesis to alter this site and evaluate the activity. This
residue was mutated to Ala and Lys to assess the requirement of
this hydrogen bonding interaction for binding naphthoic acids.
Unexpectedly, the Arg11Ala mutant still effectively methylated
naphthoic acid 3 (Table 2), although the results showed a
doubling of $K_M$ that was countered by a similar increase in $k_{cat}$.
The Arg11Lys mutant had 110% of the catalytic efficiency
compared to the WT. This mutant likewise showed an increased
rate of turnover. This observation suggests that the specific

**FIGURE 2:** Cartoon representations of NcsB1. (A) Dimer of NcsB1 with the active site region indicated. Chain A is colored light blue and chain B
yellow. Ligands are depicted in stick format with SAH colored red and naphthoic acid 4 cyan. (B) Close-up view of the monomer with secondary
structural elements and hinge regions labeled. (C) Overlay of the NcsB1–SAH–4 monomer (light blue) and the NcsB1–SAH monomer (red).
hydrogen bond from Arg11 is not entirely crucial for substrate turnover and can be offset by a similar interaction with Lys or by an increased overall rate of catalytic turnover. In addition, simple benzoic acids lacking a hydroxyl group adjacent to the aryl acid [for example, 3-hydroxybenzoic acid (see Figure S4)] were methylated by NcsB1, although at a significantly decreased rate, further suggesting that the 1,2-hydroxy acid is not absolutely required for enzyme-catalyzed chemistry.

**NcsB1 Residues Involved in Methyltransferase Chemistry.**

Roles for residues in the active site relevant for catalysis

---

**FIGURE 3:** Sequence alignment of NcsB1 structural homologues as determined by the DALI structural alignment server. Abbreviations used with accession codes from GenBank in parentheses: DnrK, *S. poacearum* O-MTase (Q06528); RdmB, *S. purpurascens* hydrolase (Q54527); IOMT, alfalfa O-MTase (Q25429); NPOMT, *N. punctiforme* putative O-MTase (ZP_00112478); PhzM, *P. aeruginosa* N-MTase (Q9HWU2); COMT, alfalfa O-MTase (P28002). Completely conserved residues are highlighted in yellow, highly conserved residues in blue, and conserved residues in green. Catalytic residues are denoted with asterisks, and the conserved glycine region is boxed.
could include activation of the phenol for nucleophilic attack via acid–base chemistry. Histidine residues specifically have been implicated to play this role in RebM, a carbohydrate O-MTase in rebeccamycin biosynthesis, where mutation of two histidine residues in the substrate binding pocket to alanines led to a complete loss of activity, while single mutations showed a marked decrease in activity (32). In NcsB1, three residues are in the proximity of the substrate phenol; the diad of His246 and Asp247 side chains and the backbone carbonyl of Ala243 may function to aid in activation and/or proton shuttling (Figure 4B).

**Binding of 1,4-Dihydroxy-2-naphthoic Acid in the Active Site of NcsB1.** Previous exploration of the substrate specificity of NcsB1 suggested a large degree of flexibility for naphthoate binding (21). A model was proposed that involved anchoring of the substrate by the 1,2-hydroxy acid motif and subsequent methylation of exposed phenols in the proximity of bound SAM. To probe this hypothesis, we determined the structure of NcsB1 bound to 1,4-dihydroxy-2-naphthoic acid (DHN, 6).

The methyl group on SAM is ∼2.1 Å from the 5-hydroxyl group of 6, and the carboxylate is hydrogen bonded to Arg11 via an ordered water (Figure 5B).

**Table 2: Kinetics of NcsB1 and Mutant Constructs**

<table>
<thead>
<tr>
<th>protein–substrate</th>
<th>$K_M$ (μM)</th>
<th>$k_{cat}$ (min⁻¹)</th>
<th>$k_{cat}/K_M$ (relative to WT/3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT–3 (1/8)</td>
<td>206 ± 49</td>
<td>0.69 ± 0.05</td>
<td>1</td>
</tr>
<tr>
<td>Tyr293Ile–3</td>
<td>649 ± 9</td>
<td>0.76 ± 0.04</td>
<td>0.35</td>
</tr>
<tr>
<td>Arg11Trp–3</td>
<td>400 ± 68</td>
<td>0.60 ± 0.03</td>
<td>0.45</td>
</tr>
<tr>
<td>Arg11Ala–3</td>
<td>419 ± 61</td>
<td>1.24 ± 0.06</td>
<td>0.88</td>
</tr>
<tr>
<td>Arg11Lys–3</td>
<td>319 ± 40</td>
<td>1.18 ± 0.05</td>
<td>1.10</td>
</tr>
</tbody>
</table>
nesB1 (56% identical, 67% similar), and the enzyme is proposed to methylate a benzoazolinate (Figure S3) (6, 48). Of nine residues in NesB1 identified to play a role in substrate binding, four have identical counterparts in SgcD4 (Trp96, Phe146, Met286, and Met290), two are similar (Met150NesB1 is a Leu in SgcD4, and Phe294NesB1 is a Tyr), and two residues are distinct (Arg11NesB1 is a Trp and Tyr293NesB1 is an Ile). To probe the substrate specificity determinants of NesB1, we introduced two rational mutations at the residues that significantly differed structurally between NesB1 and SgcD4: Tyr293Ile and Arg11Trp. These two additional mutant constructs were assayed against the natural substrate (3) (Table 2). Both mutants methylated 3, though they showed significant increases in $K_M$ compared to that of the wild-type enzyme. A larger decrease in catalytic efficiency was seen with the Tyr293Ile mutant, which had a $K_M$ approximately 3 times higher than that of the wild type. The $K_M$ was doubled for Arg11Trp. The ability of Arg11 mutants, especially the Trp and Ala mutants, to methylate naphthoic acid suggests that the aromatic binding pocket of NesB1 is substantially more crucial to binding substrate than the hydrogen bond from Arg11. The reduced aromatic character of the Tyr293Ile mutant supports this theory. We next tested a simple substrate analogue of the benzoazolinate, 2,5-dihydroxybenzoic acid. In this simple model compound, methylation occurs at the 5 position, although with significantly lower efficiency (Figure S3). 2,5-Dihydroxybenzoic acid was assayed with the panel of mutants in Table 2, and indeed, alternate specificity was observed as compared to wild-type NesB1 (Table S3). For example, although the relative measured activities were low, contrary to that of the wild-type enzyme, the Arg11Trp mutant has higher relative activity toward 2,5-dihydroxybenzoic acid and the Arg11Lys was less efficient. These results implicate Arg11 in substrate discrimination for these related methyltransferases.

In summary, we have determined the crystal structures of NesB1 in two conformations with SAH/SAM bound, with and without a substrate analogue or product. These structures revealed a large displacement of the C-terminal domain, a movement that likely opens up the active site for naphthoate binding. Additionally, the ternary complex structure of 1,4-dihydroxynaphthoic acid and SAH bound to NesB1 was determined and exhibited a rotation of this alternate substrate in the binding pocket, allowing for methylation of the hydroxyl group at the 4 position. These results led us to probe substrate binding using active site mutants, demonstrating altered substrate specificity and revealing the importance of key residues in substrate binding.

ACKNOWLEDGMENT

We thank Tim Montavon for critical reading of the manuscript and members of the Bruner and Shen groups for helpful discussions.

SUPPORTING INFORMATION AVAILABLE

Crystalization conditions (Table S1), determination of the biologically active unit of NesB1 (Figure S1), a table containing results from the DALI structural homologue search (Table S2), electron density maps (Figure S2), alignment of NesB1 with SgcD4 (Figure S3), and detailed methods and results for benzoic acid substrates (Figure S4 and Table S3). This material is available free of charge via the Internet at http://pubs.acs.org.

REFERENCES