The chondramides are mixed non-ribosomal peptide/polyketide secondary metabolites produced by the myxobacterium *Chondromyces crocatus* Cm c5, which exhibit strong cytotoxic activity. On the basis of their striking structural similarity to the marine depsipeptides jaspamides, the chondramides have been assumed to incorporate a \((R)-\beta\)-tyrosine moiety, an expectation we confirm here. Thus, the recent sequencing of the chondramide biosynthetic gene cluster provided the opportunity to probe the shared origin of this unusual \(\beta\)-amino acid. We demonstrate here that \((R)-\beta\)-tyrosine is produced directly from \(L\)-tyrosine by the aminomutase CmdF. Along with the tyrosine aminomutase SgcC4 from the C-1027 enediyne pathway, this enzyme belongs to a novel family of tyrosine aminomutases related to the ammonium lyase family of enzymes but exhibits opposite facial selectivity for the hydroxycinnamate intermediate. We also show that the adenylation (A) domain in the chondramide pathway, which activates the \(\beta\)-tyrosine building block, exhibits the required preference for \((R)-\beta\)-tyrosine, further arguing against alternative origins for the moiety in the chondramides. Comparison to the \((S)-\beta\)-tyrosine specific A domain SgcC1 should enhance our understanding of the structural and stereoselective determinants guiding amino acid selection by non-ribosomal peptide synthetase multienzymes.

Non-ribosomally synthesized peptides constitute a medicinally important class of structurally diverse natural products from bacteria. Their biosynthesis is typically performed by the stepwise condensation of amino acid building blocks on large multimodular proteins called non-ribosomal peptide synthetases (NRPS). Within each NRPS module, individual adenylation (A) domains are responsible for activating the amino acid substrates, which are then tethered as thioesters to the integral peptidyl carrier proteins (1, 2). Peptide bond formation is accomplished by the component condensation (C) domains present in each module. Chain extension is ultimately terminated by a thioesterase domain located at the end of the assembly lines.

In addition to the 20 standard amino acids, many NRPS systems incorporate non-proteinogenic amino acids into their products. Notable among these are the \(\beta\)-amino acids, which although rare in nature, are found in a number of naturally occurring NRP metabolites produced by marine organisms and terrestrial prokaryotes (3–7). These natural products not only exhibit a diverse range of biological activities, but also increased resistance to proteolytic enzymes (8, 9). Thus, attempts to tailor the pharmacological properties of NRP metabolites by genetic engineering may benefit from studies of A domains specific for \(\beta\)-amino acids as well as the pathways by which these unusual amino acids are formed in bacteria (10–13).

The aminomutase SgcC4 from the gene cluster responsible for biosynthesis of the enediyne antibiotic C-1027 in *Streptomyces globisporus*, catalyzes the conversion of \(L\)-tyrosine to \((S)-\beta\)-tyrosine, which is then further transformed to \((S)-3\text{-chloro}4,5\text{-dihydroxy}-\beta\text{-phenylalanine} (13). SgcC4 does not exhibit sequence similarity to known classes of aminomutases, but instead to phenylalanine and histidine ammonium lyases (13). Members of this family contain a signature Ala-Ser-Gly motif, which rearranges to the prosthetic group, \(4\)-methylidene-imidazole-5-one (MIO) (14). MIO acts as the catalytic electrophile in the enzyme active site, promoting the difficult \(\alpha,\beta\) elimination of ammonia from the amino acid (15). This elimination also constitutes the first half of the SgcC4-catalyzed transformation, but instead of releasing the resultant ammonia and \(\alpha,\beta\)-unsaturated acid as free products, the enzyme catalyzes Michael addition between the enzyme-bound intermediates, affording the \(\beta\)-amino acid as an end product. The shared evolutionary origin of the lyases and SgcC4 is strongly supported by the fact that SgcC4 also possesses ammonium lyase activity, generating free \(p\)-hydroxycinnamic acid (pHCA) as a minor product during catalysis. SgcC4 also exhibits an unexpected \(\beta\)-tyrosine racemase activity, possibly because its evolutionary conversion into an aminomutase from a lyase is not complete (14). Thus, although the \((S)-\beta\)-tyrosine is the kinetically preferred product of SgcC4, the enzyme can efficiently add ammonia to either face of the planar pHCA intermediate (14). Chondramides A–D (Fig. 1A), a family of molecules which...
potently affect the function of the actin cytoskeleton, contain both polyketide and polypeptide building blocks, including a β-tyrosine or α-methoxy β-tyrosine moiety (16, 17). These metabolites exhibit striking structural similarity to the jaspamides, a family of depsipeptide natural products derived from the marine sponge *Jaspis* (3), suggesting a common biosynthetic origin. In the jaspamides, the configuration of the β-tyrosine moiety is (R), indicating that the stereochemistry of the corresponding residue in the chondramides could also be (R). As these are to our knowledge the first secondary metabolites known to incorporate (R)-β-tyrosine, the origin of this moiety is unclear. Thus we aimed to take advantage of the recently sequenced chondramide gene cluster from *Chondromyces crocatus* Cm c5 (18) to elucidate the biosynthetic pathway to (R)-β-tyrosine and to determine its mode of incorporation into the natural product.

**EXPERIMENTAL PROCEDURES**

**Determination of the Configuration of β-Tyrosine in Chondramide D**—1.5 mg of chondramide D was hydrolyzed in 1.5 ml of HCl (6 N) at 99 °C for 12 h. The solvent was removed by evaporation, and then 150 μl of borate buffer (0.4 M, pH 10.2) was added to the solid residue and the suspension clarified by centrifugation. A 50-μl aliquot of the supernatant was subjected to chiral derivatization with *o*-phthalaldehyde-*N*-acetyle-lysine (OPA-NAC) reagent (19). HPLC separation of the reaction mixture was performed on a Synergy HydroRP column (150 × 2 mm, 4-μm particle size; Phenomenex Ltd., Aschaffenburg, Germany). The solvent system consisted of 20 mM ammonium formate (pH 6) (mobile phase A) and a 5% (v/v) mixture of acetonitrile and solution A (mobile phase B). The diastereomeric β-tyrosine derivatives were separated with a gradient of 25–35% B (Agilent 1100 series solvent delivery system) and detected with a ESI-MS Bruker HCT™ Plus mass spectrometer operating in manual MS/MS mode using positive ionization. OPA-NAC derivatives of commercially available (R)- and (S)-β-tyrosine (Peptech Corp., Burlington, MA) served as reference standards and were identified at retention times of 6 min (R) and 8 min (S) by their characteristic fragment ions (m/z = 314, 279, and 150, generated from the monoprotonated parent ion of m/z [β-tyrosine + OPA-NAC + H]⁺ = 443).

**Inactivation of cmdF**—An internal 1061 bp fragment of the cmdF gene including a 5′ frameshift causing base pair (shown in italics), was generated by PCR using primers TAM-frame-up (5′-CTC CAA CCT GTC CCA TCT AC-3′) and TAM-del-down (5′-GAT GTT CAG GTA GTC GCA G-3′). The fragment was cloned into the pCR2.1TOPO vector (Invitrogen) and the sequence confirmed by sequencing using primers M13uni (5′-TGT AAA ACG ACG GCC AGT-3′) and M13rev (5′-CAG GAA ACA GCT ATG ACC-3′). The gene was excised as a HindIII-EcoRV fragment, and subcloned into vector pSUPHyg (18), creating the TAM inactivation plasmid, pSR10. The plasmid was then introduced into Cm c5 cells using biparental mating with Escherichia coli strain ET12567, as described previously (18). Following selection on Pol0.3 agar supplemented with 100 μg/ml hygromycin, putative cmdF mutants were analyzed by PCR amplification of the integration region using primers TAM-frame-up and pSUP-EV (5′-GCATAT-GCGCTAGCGAC-3′ (this primer anneals to a region 53 bp downstream of the EcoRV cloning site of the pSUPHyg plasmid). Cells of the cmdF mutant strains were grown in 50 ml of MD1 medium supplemented with 100 μg/ml hygromycin for DNA isolation. Further verification of gene inactivation was performed by Northern blot analysis of cmdF transcription, essentially as described previously (20, 21). Briefly, total RNA from the Cm c5 WT and cmdF mutant strains was extracted by the TRizol (Invitrogen) method according to the manufacturer’s recommendations. A 1060 bp internal fragment of cmdF was generated by PCR using primers TAM-frame-up (5′-CTC CAA CCT GTC CAT CTA C-3′) and TAM-del-down (5′-GAT GTT CAG GTA GTC GCA G-3′), labeled with digoxigenin using the DIG-High Prime kit (Roche), and then used as a probe in the Northern blot. Hybridization was performed at 42 °C in formamide buffer with stringent washing at 68 °C followed by detection using CDP-Star (Roche Diagnostics) as substrate. As an internal control, the same northern membrane was hybridized with a ~1.2 kb DIG-labeled PCR fragment of 16S rDNA from *C. crocatus* Cm c5 using the oligonucleotides MyxordNA-F (5′-AAA GCC TGA CGC AGC-3′) and MyxordNA-R (5′-CCT ACG GCT ACC TTG-3′). For analysis of chondramide production, the wild type and cmdF mutant strains were inoculated into 100 ml of Pol03 medium supplemented with 1% XAD16 adsorber resin (Rohm & Haas, Frankfurt/Main, Germany) and 100 μg/ml hygromycin, and grown for 7 days at 30 °C in a rotary shaker at 180 rpm (Infors). The cells and resin were pelleted by centrifugation, extracted with methanol, and concentrated in vacuo. HPLC-MS analysis was performed on a reversed-phase Nucleodur C-18 column (125 × 2 mm/3 μm particle size; Macherey & Nagel), using an Agilent 1100 series instrument connected to a Bruker HCTplus ion trap.

**Complementation of the cmdF Mutant**—Complementation of the cmdF mutant was performed by growing a cmdF pre-culture in 40 ml of Pol03 medium supplemented with 100 μg/ml hygromycin for 7 days at 30 °C. The bacteria were subsequently inoculated into five conical flasks (250 ml) each containing 50 ml of Pol03 medium supplemented with 100 μg/ml hygromycin. To these production cultures, (R,S)-β-tyrosine, (R)-β-tyrosine, (S)-β-tyrosine, and L-α-tyrosine were separately added in three portions (after 24, 48, and 72 h) to a final concentration of 1 mM. 1% XAD16 resin was then added to each culture and incubation continued for a further 96 h. Bacterial cells and resin were harvested and extracted with methanol.

**Overexpression and Purification of the Adenylation Domain CmdD-A**—The CmdD-A protein was expressed as a C-terminus fusion with glutathione S-transferase (GST), as described previously (18). *E. coli* BL21 cells transformed with pGEX-Ad-7 were grown overnight in LB medium supplemented with 100 μg/ml ampicillin at 37 °C. 0.5 ml of this pre-culture was used to inoculate 50 ml of LB medium supplemented with 100 μg/ml ampicillin. The culture was incubated at 37 °C until it reached an *A*₅₆₀ of 0.6, and then expression was induced with 0.2 mM IPTG (isopropyl-β-d-thiogalactopyranoside). Growth was continued for 2 h at 30 °C before harvesting the cells by centrifugation. GST-CmdD-A was purified by glutathione affinity chromatography, with on-column cleavage using PreScission
Protease at 4 °C, according to the manufacturer’s recommendations (Amersham Biosciences).

**ATP-[^32]PP\[\text{PPi}\] Exchange Assay**—An ATP-pyrophosphate (PPi) exchange was assayed using a previously reported procedure (22). Assays were carried out at 25 °C in 100 \(\mu\)l of total volume containing 50 mM Tris (pH 8.0), 10 mM MgCl\(_2\), 100 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 2 mM dATP, 150 nM CmdD-A7, 1 mM [^32]PPi-pyrophosphate (0.5 \(\mu\)Ci), and 2 mM of either L-\(\alpha\)-tyrosine, (R,S)-\(\beta\)-tyrosine (Johnson Pump), (R)-\(\beta\)-tyrosine, (S)-\(\beta\)-tyrosine, L-tryptophan, L-phenylalanine, or L-methionine as substrates. Determination of the apparent \(K_m\) value for (R)-\(\beta\)-tyrosine was performed by addition of 0.2, 0.25, 0.5, 1.0, and 2.0 mM (R)-\(\beta\)-tyrosine to the reactions. After a 15-min incubation period at 25 °C, the reactions were quenched by addition of an aqueous mixture of 1.6% (w/v) activated charcoal, 4.46% (w/v) tetrasodium pyrophosphate, and 3.5% perchloric acid. The charcoal pellet was washed twice with the quenching mixture and submitted for liquid scintillation counting using a Packard BioScience Tri-Carb 2900 TR liquid scintillation counter. The reactions were typically performed in triplicate. The activation was plotted against substrate concentrations and fit to the Michaelis-Menten equation by direct non-linear regression using the enzyme kinetics module of the SigmaPlot software suite (Systec Software Inc., San Jose, CA).

**Recombinant Production and Purification of CmdF**—CmdF was expressed as a C-terminal fusion to GST. PCR amplification of the cmdF gene from chromosomal DNA of \(Cm\) c5 was carried out using Pfu polymerase (Stratagene) and the oligonucleotides TAM-BamHI-up (5'-CGG ATC CAT GAA AAT CAC TGG CTC CAA-3') (the BamHI site is underlined) and TAM-EcoRI-down (5'-AGG AAT TCC TCA GGA TGA CGC GGT CGT-3') (the EcoRI site is underlined). The resulting 1612 bp product was purified, and single A overhangs were added using Taq polymerase. The fragment was cloned into the pCR2.1TOPO vector (Invitrogen), and the fidelity of the PCR amplification was verified by sequence confirmation.

**FIGURE 1.** Structure of the chondramides A–D and HPLC-MS analysis of a chondramide D hydrolysate following chiral derivatization. A, chondramide A \(\{R_1 = OCH_3, R_2 = H\};\) chondramide B \(\{R_1 = OCH_3, R_2 = Cl\};\) chondramide C \(\{R_1 = R_2 = H\};\) chondramide D \(\{R_1 = H, R_2 = Cl\}\). B, HPLC-MS analysis revealed that the molecule contained exclusively (R)-\(\beta\)-tyrosine \(\{a\}\). Minor amounts of the respective opposite enantiomers were also found in the commercial reference compounds, (R)-\(\beta\)-tyrosine \(\{b\}\) and (S)-\(\beta\)-tyrosine \(\{c\}\). Trace \(\{d\}\) is the derivatized mixture of both \(\beta\)-tyrosine enantiomers. The extracted ion chromatograms are shown.

**FIGURE 2.** Multiple sequence alignment of CmdF from \(C.\) crocatus (GenBank™ entry AM179409) with the biochemically characterized tyrosine aminomutase SgcC4 from \(S.\) globisporus (GenBank™ entry AAL06680) and the histidine ammonium lyase Huth from \(Pseudomonas\) putida (GenBank™ entry AAN70597). The highly conserved Ala-Ser-Gly residues that form the active site MIO group through autocatalytic post-translational modification are marked with asterisks. The residue at position 431 (\{\}\) numbering from CmdF) is almost invariably glutamic acid in ammonium lyases specific for histidine, and glutamine in lyases which act on phenylalanine or tyrosine. It can therefore be used reliably for in silico prediction of substrate specificity.
Incorporation of (R)-β-Tyrosine into the Chondramides

analysis. The insert was excised using restriction enzymes BamHI and EcoRI and subcloned into expression vector pGEX-6P-1 (Amersham Biosciences) previously digested with BamHI and EcoRI, creating the expression plasmid pGEX-TAM. *E. coli* BL21 carrying the plasmid pGEX-TAM was used to inoculate 2 ml of LB medium containing 100 µg/ml ampicillin and grown overnight at 37 °C. The preculture was inoculated into 50 ml of LB medium supplemented with ampicillin at a 1:100 dilution and grown at 37 °C until an A600 of 0.6. 0.5 mM IPTG was added, and cultivation was continued for 6 hr at 25 °C. Cells were harvested by centrifugation at 6500 × g for 15 min at 4 °C, and the pellet was resuspended in phosphate-buffered saline. Cells were passed twice through a French pressure cell at ~18000 psi, and the lysate centrifuged at 12,000 × g for 30 min at 4 °C. Protein purification was carried out by affinity chromatography using GST-Sepharose, and the GST tag was cleaved with PreScission Protease at 4 °C according to the manufacturer’s recommendations (Amersham Biosciences). The purified protein was eluted in buffer (50 mM Tris-HCl (pH 7.0), 150 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol) and stored in aliquots at −80 °C. Protein concentration was determined using the method of Bradford (23).

**Assay in Vitro for CmdF Aminomutase Activity**—CmdF enzyme activity was assayed using 850 µg of purified protein in 0.1 M Tris buffer (pH 8.8) at 30 °C, using 2 mM L-α-tyrosine, as described previously (14). To investigate the formation of β-tyrosine, a 50-µl aliquot from the assay was derivatized with OPA-NAC reagent (19). Briefly, an aliquot of the assay was mixed with 200-µl reagent for 10 min at room temperature and directly submitted to HPLC analysis as described in (24). Retention times for the amino acid OPA-NAC derivatives were l-α-tyrosine, 16.5 min; (R)-β-tyrosine, 12 min; (S)-β-tyrosine, 15 min.

**RESULTS AND DISCUSSION**

(R)-β-Tyrosine Is Incorporated into the Chondramides—Comparison of the chondramides to the known structures of the jaspadines (3) suggested that the configuration of the β-tyrosine in the chondramides should be (R). To confirm this, however, hydrolysis of chondramide D was performed by the addition of HCl. HPLC-MS-MS analysis of the chondramide D hydrolysate following chiral derivatization showed unambiguously that (R)-β-tyrosine was incorporated into the molecule (Fig. 1B). This experiment also revealed that the commercially available (R)- and (S)-β-tyrosines used in this study both contained trace amounts of the opposite enantiomer.

**Inactivation of the Tyrosine Aminomutase Gene and Complementation Studies**—The biosynthesis and incorporation of (S)-β-tyrosine has been investigated in detail for C-1027 biosynthesis (12, 25), but to our knowledge, there is no information on the origin of the (R)-β-tyrosine building block found in the chondramides and jaspadines. We hypothesized, however, that (R)-β-tyrosine could be produced directly from l-tyrosine by a tyrosine aminomutase similar to SgcC4 (13). Indeed, sequence analysis of the downstream region of the cmdA – cmdD operon revealed the presence of a gene, cmdF, whose gene product showed convincing homology to SgcC4 (41% identity and 57% similarity on the amino acid level). Neither gene exhibited homology to the known class of aminomutase enzymes including radical-based enzymes and ATP- or 4-pyridoxalphosphate dependent enzymes (26–28), but both are homologous to a large number of histidine ammonium and phenylalanine lyases of prokaryotic origin. CmdF also contains the signature Ala-Gly motif characteristic of the ammonium lyases and thus is likely to use the prosthetic MIO group in catalysis (Fig. 2) (15). In addition, both CmdF and SgcC4 incorporate a strictly conserved glutamine residue (Gln-431 in CmdF and Gln-442 in SgcC4), which is characteristic of ammonium lyases which use phenylalanine or tyrosine as substrates (24, 29). Taken together, these data suggested that CmdF was likely to be a tyrosine aminomutase, belonging to the ammonium-lyase family. Thus, we evaluated its putative role in the provision of (R)-β-tyrosine by targeted gene inactivation.

Inactivation of the cmdF gene was achieved via integration by homologous recombination of the inactivation plasmid pSR10 into the genome of the wild type Cm c5 strain. Polar effects on other genes in the cluster were judged unlikely, as cmdF appeared to be located at the end of an operon (Fig. 3, A and B). Verification of the cmdF− mutants was obtained by PCR using primer TAM-del-up and vector-specific primer pSUP-EV; a 1135 bp fragment was amplified from genomic DNA of the cmdF− mutant, whereas no PCR fragment was generated from wild type genomic DNA. Furthermore, the absence of the cmdF transcript from total RNA of the cmdF− mutant strain was confirmed by Northern blot (Fig. 3C). HPLC-MS analysis of methanolic extracts from production cultures of the cmdF− mutant revealed that the strain no longer produced the chondramides (Fig. 3, D–G) (data for the wild type compounds: chondramide A (Rt = 15.9 min, m/z [M + H]+ = 647); chondramide C (Rt = 16.7 min, m/z [M + H]+ = 617); chondramide B (Rt = 16.9 min, m/z [M + H]+ = 681); chondramide D (Rt = 18.0 min, m/z [M + H]+ = 651)) (18).

We next attempted to complement the cmdF− mutant by addition of 1 mM racemic (R,S)-β-tyrosine, (R)-β-tyrosine, (S)-β-tyrosine, and l-α-tyrosine to the production cultures. This choice of substrates allowed us simultaneously to probe the stereochemistry of β-tyrosine incorporation into the chondramides. HPLC-MS analysis of methanolic extracts showed that chondramide production was restored only when (R,S)-β-tyrosine (data not shown) or (R)-β-tyrosine were supplied (Fig. 4). Chondramides were not observed in the cultures following

**FIGURE 3. Inactivation of the cmdF gene by integration of the pSR10 plasmid. A, physical map of the chondramide gene cluster (18). B, insertion of the cmdF inactivation plasmid pSR10 into the Cm c5 WT chromosome via single crossover. C, Northern blot analysis of total RNA from C. crocatus Cm c5 (lane 1) and C. crocatus cmdF− (lane 2) hybridized with a DIG-labeled internal fragment of the cmdF gene. The Northern blot of the 16 S rRNA control, shown in the lower panel, was hybridized with a DIG-labeled PCR product amplified from the 16 S rRNA of Cm c5. D, HPLC-MS chromatogram of the Cm c5 WT methanolic extract showing the chondramides. Peak 1, corresponding to chondramide A, MW [M + H]+ = 647.3; peak 2, chondramide C, MW [M + H]+ = 617.3 and chondramide B, MW [M + H]+ = 681.3; peak 3: chondramide D, MW [M + H]+ = 651.2; E, HPLC-MS analysis of the cmdF− mutant. F, mass spectrum of peak within chromatogram D at 16.8 min. G, mass spectrum of peak within chromatogram E at 16.8 min.**
addition of L-tyrosine, but a small amount of chondramide D was detected upon supplementation with (S)-β-tyrosine (Fig. 4, B and F). However, we attribute biosynthesis of this metabolite to the presence of trace amounts of (R)-β-tyrosine in the commercial sample of (S)-β-tyrosine (Figs. 1B and 4). As inactivation of CmdF resulted in the loss of chondramide production, and the mutation could be complemented by addition of (R)-β-tyrosine, our results implied that CmdF is directly responsible for biosynthesis of this building block.

**The CmdF Protein Converts L-Tyrosine to β-Tyrosine in Vitro**

To confirm the putative activity of CmdF in vitro, CmdF was overexpressed in *E. coli* BL21 as C-terminal fusion with GST. Purification of CmdF by glutathione affinity chromatography, followed by on-column cleavage using PreScission Protease, yielded ~3 mg/liter of culture. The purified CmdF protein (calculated *M*<sub>c</sub>, 57309 Da) migrated during SDS-PAGE analysis with the expected size (Fig. 5A).

The purified enzyme was assayed for aminomutase activity by incubation of CmdF with L-tyrosine, using conditions previously reported for the investigation of aminomutase and ammonium-lyase-type enzymes (14). Products were detected using diode array detector-coupled HPLC separation following chiral derivatization, and product identity was verified by comparison to commercially available reference compounds (see under “Experimental Procedures”). Analysis of assay samples taken at multiple time points during a 22-h incubation revealed that CmdF was able to convert L-α-tyrosine into β-tyrosine in the absence of added cofactors (Fig. 5B). The primary product of the reaction was (R)-β-tyrosine, but (S)-β-tyrosine was produced at later time points. Formation of pHCA was also observed, consuming about 30% of the substrate L-tyrosine in the course of the above assay (data not shown). No activity was observed with D-tyrosine or L-histidine as substrates. As (R)-β-tyrosine is the kinetically preferred product of CmdF, these data confirm our expectation that the enzyme can produce (R)-β-tyrosine directly from the precursor L-tyrosine.

Taken together, these observations suggest that CmdF and SgcC4 accomplish the synthesis of β-tyrosine using a common catalytic mechanism (14), and therefore, that CmdF represents the second member of a novel class of aminomutase enzymes. The essential difference between the enzymes is the facial selectivity, with CmdF generating (R)-β-tyrosine and SgcC4 producing (S)-β-tyrosine from a common intermediate. The basis for the control of stereochemistry during ammonia addition is
presently unclear but should be illuminated by high-resolution structural information on these enzymes.

The CmdD-A<sub>7</sub> Adenylation Domain Preferentially Activates (R)-β-Tyrosine—The A domains of NRPS multienzymes are responsible for selection of the amino acid incorporated by each module in the NRPS. Therefore, if the (R)-β-tyrosine of CmdF is directly incorporated into the chondramides, the A domain in the respective module (CmdD module 7, Fig. 6) should exhibit a strong preference for this substrate. Similar studies have recently been performed with the A domain SgcC1 of the C-1027 pathway, demonstrating a 25-fold preference ($k_{cat}/K_m$) for (S)-β-tyrosine over the (R) isomer (25). Therefore, we aimed directly to evaluate the specificity of recombinant CmdD-A<sub>7</sub> toward a panel of potential substrates, including both (R)- and (S)-β-tyrosine.

Recombinant production of GST-tagged CmdD-A<sub>7</sub> domain in *E. coli* BL21 was achieved using the expression plasmid pGEX-Ad-7 as described (18). The substrate specificity of the purified A domain was investigated using the standard amino acid-dependent ATP-PP<sub>i</sub> exchange assay (22). Incubation with a panel of amino acids (Fig. 7A) in the presence of [32P]-pyrophosphate showed that (R)-β-tyrosine gave the highest activity (set to 100% in Fig. 7A). Only 0.1–1% of this activity was observed in the absence of enzyme. Incubation with (R,S)-β-tirosine resulted in 24% relative activity followed by L-α-tirosine (22%), phenylalanine (15%), (S)-β-tirosine (10%), and tryptophan (6%). Activity toward methionine was essentially at background levels. By varying the amount of (R)-β-tirosine (0.2–2 mM), we estimated its $K_m$ value to be 1.2 mM (Fig. 7B) (it was not possible to further increase the (R)-β-tirosine concentration because of its poor solubility, and therefore this figure represents the maximum value for the $K_m$). This value is significantly higher than that determined for SgcC1 with (S)-β-tirosine (3.2 μM) but within the range of $K_m$ values reported for A domains toward their native substrates (25, 30). Thus, as required by the biosynthetic model, CmdD-A<sub>7</sub> exhibits a very strong preference for (R)-β-tirosine over other potential substrates. These data argue against an alternative model for chondramide formation.
biosynthesis in which CmdD-A7 activates L-tyrosine followed by transformation of the peptidyl carrier protein-bound residue to (R)-β-tyrosine.

Analysis of the specificity pockets of A domains has resulted in the identification of eight residues, which can be used to predict the activation specificity (31, 32). Comparison between these selectivity-determining residues in CmdD-A7 and SgcC1 reveals substantial divergence (DGSTITAV and DPAQLMLI, respectively), which may account for the differences both in stereospecificity toward β-amino acids and the strength of substrate binding. On the basis of multiple sequence alignment of A domains, it has also been proposed that the replacement of Ala or Gly by Pro in the second position of the specificity code may alter the specificity of the domain to favor β-amino acids (25). However, CmdD-A7 does not contain this “signature replacement”, which suggests that care must be taken in drawing conclusions from the limited available structure-activity relationships for A domains. Clearly, a crystal structure of a β-amino acid activating A domain would shed light on the molecular basis for selective activation of β-amino acids.

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