Novobiocic acid synthetase, a key enzyme in the biosynthesis of the antibiotic novobiocin, was cloned from the novobiocin producer *Streptomyces spheroides* NCIMB 11891. The enzyme is encoded by the gene *novL*, which codes for a protein of 527 amino acids with a calculated mass of 56,885 Da. The protein was overexpressed as a His6 fusion protein in Escherichia coli and purified to apparent homogeneity by affinity chromatography and gel chromatography. The purified enzyme catalyzed the formation of an amide bond between 3-dimethylallyl-4-hydroxybenzoic acid (ring A of novobiocin) and 3-amino-4,7-dihydroxy-8-methyl coumarin (ring B of novobiocin) in an ATP-dependent reaction. NovL shows homology to the superfamily of adenylate-forming enzymes, and indeed the formation of an acyl adenylate from ring A and ATP was demonstrated by an ATP-PPi exchange assay. The purified enzyme exhibited both activation and transferase activity, i.e. it catalyzed both the activation of ring A as acyl adenylate and the subsequent transfer of the acyl group to the amino group of ring B. It is active as a monomer as determined by gel filtration chromatography. The reaction was specific for ATP as nucleotide triphosphate and dependent on the presence of Mg2+ or Mn2+. Apparent Km values for ring A and ring B were determined as 19 and 131 μM, respectively. Of several analogues of ring A, only 3-gerranyl-4-hydroxybenzoate and to a lesser extent 3-methyl-4-aminoenzoate were accepted as substrates.

The aminocoumarin antibiotic novobiocin is produced by *Streptomyces spheroides* and *Streptomyces niveus*. Novobiocin (see Fig. 1) consists of three moieties: a prenylated 4-hydroxybenzoic acid (ring A),1 a substituted aminocoumarin moiety (ring B), and a deoxysugar (ring C). Ring A is attached to the amino group of ring B via an amide bond. Both aromatic rings are derived from tyrosine, ring C is derived from glucose, and the prenyl group of ring A is formed via the nonmevalonate pathway (1–3). The antimicrobial activity of novobiocin results from its interaction with bacterial DNA gyrase, which has been investigated by x-ray crystallographic studies (4–7). The detailed knowledge available about the structural elements of novobiocin involved in its binding to the biological target may permit rational approaches in the search for new aminocoumarin derivatives. Recently, the development of new, synthetic aminocoumarin compounds with gyrase-inhibiting activity has been reported (8–10). Like novobiocin itself (Albamyacin®, Pharmacia & Upjohn), such new aminocoumarins may serve as antibiotics for the treatment of infections with multi-resistant Gram-positive bacteria such as *Staphylococcus aureus* or *Staphylococcus epidermidis* (11–13).

Genetic engineering and combinatorial biosynthesis in bacteria provide an important new tool for drug discovery. Besides polyketide synthetases, peptide synthetases especially have been successfully used for such approaches (14–17). Knowledge of the sequence and function of the genes involved in the biosynthesis of natural products is a prerequisite for such research. We have recently cloned and sequenced the biosynthetic gene cluster for novobiocin from *S. spheroides* NCIMB 11891 and have assigned functions to the biosynthetic genes by comparison with GenBank™ entries and by gene inactivation experiments (18). A key step in the biosynthesis of novobiocin is the formation of the amide bond between ring A and ring B (Fig. 1) in an ATP-dependent reaction; this enzymatic reaction, termed novobiocic acid synthetase, has been demonstrated previously in crude extracts from a novobiocin-producing strain (19). A detailed investigation of this reaction is of particular interest for the development of new aminocoumarin antibiotics; whereas ring B and ring C are essential for the binding of novobiocin to gyrase, the structure of ring A can be varied without loss of antibiotic activity (10, 20). It has been suggested that the structure of ring A influences the uptake of the antibiotic through the bacterial membrane (20, 21). Cloning of the gene(s) for novobiocic acid synthetase and investigation of the substrate specificity of this reaction may therefore assist in the development of novobiocin derivatives with a modified ring A. We now report the cloning, overexpression, purification, and characterization of novobiocic acid synthetase from *S. spheroides* NCIMB 11891, encoded by the gene *novL*.

**EXPERIMENTAL PROCEDURES**

**Chemicals and Radiochemicals**

[Tetrasodium pyrophosphate (126.4 Gbq/mmol)](http://www.jbc.org/) was obtained from NEN Life Science Products. Ring B and novobiocic acid were kindly provided by Pharmacia & Upjohn, Inc. (Kalamazoo, MI). 3-Cyclohexyl-4-hydroxybenzoic acid was a gift from L. Wessjohann (Amsterdam, Netherlands). Plicatin B was kindly provided by R. Bates (Bangkok, Thailand). Ring A was obtained by hydrolysis of novobiocin as described previously (19). 3-Dimethylallyl-4-hydroxyxycinnamic acid...
Novobiocic Acid Synthetase

**Fig. 1. Biosynthesis of novobiocin.**

was synthesized by hydrolysis of plicatin B as described in Bates et al. (22); EI-MS analysis on a TSQ70 spectrometer (Finnigan, Bremen, Germany) using methanol as solvent confirmed the identity of the product (observed molecular weight, 232.2; theoretical molecular weight of C14H16O3, 232.3). 3-Geranyl-4-hydroxybenzoic acid (GBA) was synthesized enzymatically from 4-hydroxybenzoic acid and geranyl diphosphate with 4-hydroxybenzoyl polypeptidyltransferase of Escherichia coli (23); the incubation mixture (3 ml) contained 0.4 mM 4-hydroxybenzoic acid, 2 mM geranyl diphosphate, 50 mM MgCl2, 1 mM KF, 50 mM Tris-HCl buffer, pH 8.0, and 0.2 mg/ml membrane protein fraction and was incubated for 60 min at 37 °C. The reaction was stopped by addition of 90 µl of concentrated formic acid. GBA was extracted with 30 ml of n-hexane, the organic phase was evaporated, and the residue was dissolved in 50 mM Tris-HCl buffer, pH 8.0.

**Bacterial Strains, Cloning Vectors, and DNA Manipulations**

The bacterial strains and plasmids used in this study are listed in Table I. Plasmid pUWL201 was kindly provided by A. Bechthold (Tübingen, Germany) and originally obtained from U. Wehmeier (Wupper- tal, Germany). Cloning experiments were performed in *E. coli* XL1 Blue MRF by standard procedures (24). Heterologous expression experiments with *Streptomyces lividans* TK24 were carried out as described previously (18). Enzyme activity was determined after cultivation in CDM medium (20 µg/ml thiotrepton) for 3–4 days at 28 °C and 170 rpm in baffled shake flasks. DNA manipulations and standard genetic techniques in *E. coli* and *Streptomyces* species were carried out as described in Sambrook et al. (24) and Hopwood et al. (25).

**Construction of pMS80 for Expression of NovL as Fusion Protein with a C-terminal HisTag**

**Construction of pMS82 for Expression of NovL as Fusion Protein with an N-terminal HisTag**

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**Construction of pMS80 for Expression of NovL as Fusion Protein with a C-terminal HisTag**

**Construction of pMS82 for Expression of NovL as Fusion Protein with an N-terminal HisTag**

was amplified by PCR using pMS76 DNA as template. An SphI site was introduced at the place of the natural start codon, using primer novL-1 (5’-TAGCCACGGCATGCGAACAAGGATCAC-3’); bold letters represent the SphI site. At the C terminus, a BamHI site was introduced before and an EcoRI site behind the stop codon, using primer novL-3 (5’-CATCGAATTTCAGAGATCCCTGTCACCA-3’); bold letters represent introduced restriction sites). The PCR mixture (100 µl) contained 100 ng of pMS76 template, 22 pmol of each primer, 0.2 mM dNTPs (Stratagene), Pfui DNA polymerase reaction buffer, and 5% (v/v) MeSO. 25 units of cloned Pfui DNA polymerase (Stratagene) were added after an initial denaturation for 5 min at 96 °C, followed by 27 cycles (95 °C for 90 s, 72 °C for 45 s, and 72 °C for 4 min). The PCR product was digested with SphI and BamHI before ligation into the same sites of the expression vector pQE70, resulting in a C-terminal in-frame fusion with the His-tag of pQE70. The resulting plasmid was designated as pMS80.

**Expression of pMS80 and Purification of Novobiocic Acid Synthetase**

**Expression of pMS80 and Purification of Novobiocic Acid Synthetase**

*E. coli* XL1 Blue MRF was used as host for the expression of pMS80. Cells were cultured at 30 °C in LB medium (24) supplemented with 50 µg/ml carbenicillin until an *A*<sub>600</sub> of 0.7 was reached. 0.5 mM isopropyl-β-D-thiogalactoside (IPTG) was added, and after further growth for 3 h at 30 °C, cells were harvested by centrifugation and washed with 50 mM Tris-HCl, pH 8.0. All subsequent steps were carried out at 4 °C. Cells (3 g) were suspended in 3 ml of lysis buffer (50 mM NaH<sub>2</sub>PO<sub>4</sub>, pH 8.0, 300 mM NaCl, 10 mM imidazole, 1 mg/ml lysozyme). After incubation on ice for 30 min, the cell suspension was sonicated for 1 min (Branson Sonifier B-275). The homogenate was loaded onto a column containing 5 ml Ni-NTA-agarose slurry (50% (w/v) nickel-nitrilotriacetic acid agarose in 30% (v/v) ethanol). The mixture was incubated on ice for further 10 min. After removal of the cell debris by centrifugation (17,500 × g for 30 min), 1 ml of Ni-NTA-agarose slurry (50% (w/v) nickel-nitrilotriacetic acid agarose resin suspension in 30% (v/v) ethanol, precharged with Ni<sup>2+</sup>) (Qiagen) were added and mixed gently by shaking for 60 min. The lysate-Ni-NTA-agarose mixture was loaded onto a column containing 5 ml Ni-NTA-agarose slurry. The column was washed with 8 ml of wash buffer (50 mM NaH<sub>2</sub>PO<sub>4</sub>, pH 8.0, 300 mM NaCl, 20 mM imidazole). The Ni-NTA-agarose eluate was applied to a HiLoad 26/60 Superdex 200 column (Amersham Pharmacia Biotech) that had been equilibrated with 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 5 mM diithiothreitol, and 50 µM phenylmethylsulfonyl fluoride. Chroma-
Novobiocic Acid Synthetase

| Strain or plasmid | Description | Reference or source |
|-------------------|-------------|---------------------|
| *S. spheroides* NCIB 11891 | Wild type, novobiocin producer | NCIMB |
| *S. lividans* TK24 | Streptomycin-resistant, no plasmids | Ref. 25 |
| E. coli XLI Blue MRF’ | Tet’ | Stratagene |
| E. coli BL21 (DE3)pLysS | Cam’ | Novagen |
| pBluescript SK(−) (pBSK(−)) | Amp’ | Stratagene |
| pGEM-11zf (+) | Amp’ | Promega |
| pQE70 | T5 promoter, C-terminal His<sub>6</sub> tag, Amp’ | QIagen |
| pSet B | T7 promoter, N-terminal His<sub>6</sub> tag, Amp’ | Invitrogen |
| pUWL201 | Amp’ Tsr‘ ermE up promoter | U. Wehmeier* |
| p9–6GE9 | 9.7-kb EcoRI fragment from comid 9–6G of *S. spheroides* cosmid library in pBSK(−) | Ref. 18 |
| pMS65 | 1.95-kb EcoRI-BglII fragment (novH) of p9–6GE9 in pUWL201 | Ref. 18 |
| pMS73 | 6.96-kb EcoRI-XbaI fragment (novH, I, J, K, and L) of p9–6GE9 in pUWL201 | Ref. 18 |
| pMS76 | 2.1-kb Apol fragment (novL) of p9–6GE9 in pGEM-11zf (+) | This work |
| pMS77 | 2.1-kb EcoRI-XbaI fragment (novL) of pMS76 in pUWL201 | This work |
| pMS78 | 1.95-kb EcoRI-BglII fragment of p9–6GE9 in pMS76 (novH and novL) | This work |
| pMS79 | 4.08-kb EcoRI-XbaI fragment (novH and novL) of pMS78 in pUWL201 | This work |
| pMS80 | novL in pQE70 | This work |
| pMS82 | novL in pSet B | This work |

* Unpublished data.

**Protein Analysis**

Protein concentrations were determined by the Bradford method (26) using bovine serum albumin as a standard. SDS-PAGE was carried out according to the method of Laemmli (27), and protein bands were stained with Coomassie Brilliant Blue R-250. The molecular weight of native NovL was determined by gel filtration on a HiLoad 26/60 Superdex 200 column using the buffer described above. The column was calibrated with blue dextran 2000, aldolase (molecular weight, 158,000), albumin (molecular weight, 67,000), ovalbumin (molecular weight, 43,000), and ribonuclease A (molecular weight, 13,700) (Amer sham Pharmacia Biotech).

**Enzyme Assays**

**Novobiocic Acid Synthetase Assay**—The novobiocic acid synthetase assay contained 1 μM ring A, 1 μM ring B, 5 mM ATP, 5 mM MnCl<sub>2</sub>, and 50 mM Tris-HCl, pH 8.0, in a final volume of 100 μl. To assay the activity of crude extracts, 20–100 μg of protein and an incubation time of 20 min were used. To assay the activity of purified novobiocic acid synthetase, a maximum of 5 μg of enzyme and an incubation time of 7 min were used to ensure linearity of the product formation. The reaction was carried out at 30 °C and stopped by addition of 5 μl of 1.5 M trichloroacetic acid. The reaction mixture was extracted with 1 ml of ethyl acetate, the organic phase was evaporated, and the residue was dissolved in H<sub>2</sub>O/methanol (50:50, v/v). HPLC analysis was carried out using a Multosphere RP18–5 column (250 × 4 mm, 5 μm; C<sub>18</sub> Chromatographic Service, Düren, Germany) with a linear gradient from 60 to 100% methanol in 1% aqueous formic acid and detection at 205 nm. Authentic novobiocic acid was used as a standard.

**ATP-PP Exchange Assay**—The reaction mixture of the ATP-PP exchange assay (28) contained (in a final volume of 100 μl) 50 mM Tris-HCl, pH 8.0, 5 mM MnCl<sub>2</sub>, 5 mM ATP, 0.1 mM tetrasodium pyrophosphate, 34.4 K<sub>B</sub> (p<sup>32</sup>P)tetrasodium pyrophosphate, 4 μg of purified novobiocic acid synthetase, and 1 μl of the tested substrates ring A or ring B. After incubation for 20 min at 30 °C, the reaction was stopped by adding 1 ml of a mixture containing 1:2 (v/v) activated charcoal, 0.1 μM tetrasodium pyrophosphate, and 3% (v/v) perchloric acid. The charcoal was pelleted by centrifugation (14,000 rpm for 5 min), washed twice with 1 ml of water, and finally resuspended in 0.5 ml of water. The charcoal-bound radioactivity was measured using a Tri-Carb 2100 TR scintillation analyzer (Canberra-Packard) after addition of 9 ml of liquid scintillation fluid (Rotiszint® Eco Plus, Roth).

**Preparative Isolation and EI-MS Analysis of Enzymatic Products**

To confirm the identity of the product of the novobiocic acid synthetase reaction, 2 ml (22.5 mg of protein) of a crude extract from *S. lividans* TK24 transformed with the expression construct pMS77 were passed through a Sephadex G-25 column and incubated in 50 mM Tris-HCl, pH 8.0, with 1 mM ring A, 1 mM ring B, 5 mM ATP, and 5 mM MnCl<sub>2</sub> in a final volume of 15 ml for 60 min at 30 °C. After addition of 750 μl of 1 M trichloroacetic acid, the incubation mixture was extracted for three times with 20 ml of ethyl acetate. The organic phases were combined, evaporated, and dissolved in H<sub>2</sub>O/methanol (50:50, v/v), and the reaction product was purified by HPLC as described for the novobiocic acid synthetase assay (see above). EI-MS was carried out as described under “Chemicals and Radiochemicals” using dichloromethane as a solvent. A molecular weight of 395.2 was observed (for novobiocic acid, C<sub>22</sub>H<sub>21</sub>NO<sub>6</sub>, the molecular weight was 395.4).

**RESULTS**

**Identification of the Novobiocic Acid Synthetase Gene**—Sequence analysis of the novobiocin biosynthetic gene cluster revealed two genes for which homology searches suggested a possible involvement in the novobiocic acid synthetase reaction (18); the deduced protein sequence of *novH* showed similarity to nonribosomal peptide synthetases, and *novL* showed homology to acyl-CoA synthetases and 4-coumarate:CoA ligases. Novobiocic acid synthetase activity could be demonstrated (18) upon heterologous expression of a 6.86 kb DNA fragment comprising *novH*, *novL*, and three further complete open reading frames (Fig. 2, pMS73). To identify the genes involved in this reaction, we now prepared additional constructs containing these genes, expressed them in *E. coli* TK24, and examined the resulting novobiocic acid synthetase activity. All constructs were derived from pUWL201 and contained the erm<sup>E</sup> up promoter for foreign gene expression. Whereas expression of *novH* (Fig. 2, pMS65) yielded no activity, novobiocic acid synthetase was clearly detected upon expression of *novL* (pMS77). The identity of the enzymatic product was confirmed by HPLC in comparison with authentic substance and by preparative isolation of the product followed by mass spectrometry (EI-MS; see “Experimental Procedures”). Simultaneous expression of *novH* and *novL* (pMS79) did not increase activity in comparison to the expression of *novL* alone (Fig. 2), and likewise a mixture of the two enzyme extracts obtained after separate expression of genes *novH* and *novL* (pMS65 and pMS77), respectively, did
not show higher activity than the extract obtained from the expression of novL alone (data not shown). This demonstrates that the novobiocic acid synthetase reaction is catalyzed by NovL alone and that NovH is not required for this activity. Within the novobiocin biosynthetic gene cluster (GenBank™ accession number AF170880), novL spans positions 12457–14040. It comprises 1584 base pairs and encodes a protein of 527 amino acids (calculated mass, 56,885 Da). The coding region has an overall G+C content of 70.1%. Upstream of the GTG initiation codon, a putative ribosomal binding site (AGG-TAG) was identified. Fig. 3 shows that NovL contains several conserved motifs supposed to be involved in common steps of adenylate formation, i.e. nucleotide binding, PP_i release, and adenylation of the carboxylate moiety of the substrate. These motifs include Box I (SSGTTGXPKGV) and a sequence similar to the Box II motif (usually GEICIRG) of 4-coumarate:CoA ligases (29), as well as motifs A8 and A10 in the C-terminal domain (30). As confirmed by mutational analysis, both the conserved Lys of the Box I motif and the conserved Arg of motif A8 cooperate in coordinating the pyrophosphate release during adenylate formation (29). The conserved Lys of motif A10 interacts with the carboxyl group of the substrate as well as with the ribose oxygens O-4 and O-5 (30). Becker-André et al. (31) suggested a participation of the central cysteine within Box II motif of 4-coumarate:CoA ligases, but this hypothesis was recently disproven by mutational analysis (29). A distinction between coenzyme A ligases and enzymes that merely form acyl adenylates is therefore not possible from sequence data. In contrast to nonribosomal peptide synthetases, NovL did not show a 4'-phosphopantetheinyl attachment site.
Overexpression of the novL Gene in *E. coli*—For further characterization, NovL was expressed in *E. coli* in form of different fusion proteins with a His₈ tag for metal affinity chromatography (see “Experimental Procedures”). Fusion of the His tag to the N terminus of NovL resulted in the expression of an insoluble protein, and only minimal novobiocic acid synthetase activity (0.74 pkat/mg protein) was detectable in the soluble fraction. Fusion of the His tag to the C terminus of NovL gave approximately 7-fold higher activities (5.1 pkat/mg) (Fig. 4), most of which was still in the insoluble fraction. The amount of soluble protein could be increased, however, by decreasing the growth temperature to 30 °C, reducing IPTG concentration to 0.5 mM, induction at a later stage of the growth phase (A₆₀₀ = 0.7), and shortening of the induction period to 3 h. Under these conditions, soluble novobiocic acid synthetase was obtained in an activity of 461 pkat/mg protein.

**Purification of Novobiocic Acid Synthetase**—The fusion protein of NovL with the C-terminal His₈ tag was purified by metal affinity chromatography. Because SDS-PAGE of the eluate (Fig. 4) showed some impurities, gel chromatography on Superdex 200 was used as additional purification step and yielded a protein of apparent homogeneity (Fig. 4). The gel chromatography step also served for the removal of imidazole; imidazole was necessary for elution from the metal affinity column but greatly reduced the stability of the enzyme. After removal of imidazole and in the presence of 50 mM phenylmethylsulfonyl fluoride and 5 mM dithiothreitol, the purified enzyme could be stored at 4 °C for 2 days with only 22% loss of activity. Overall, the enzyme was purified 115-fold with an overall yield of 55% (Table II).

**Molecular Weight Determination and Characterization of NovL**—By SDS-PAGE, the molecular mass of the His-tagged protein was determined as 60 kDa (Fig. 4). By gel filtration, the native molecular mass of the enzyme resulted as 57–58 kDa (calculated mass, 58.1 kDa), showing that the protein is active as a monomer. The purified NovL exhibited a specific activity of 50.6 nkat/mg protein, corresponding to a turnover rate of 2.9 mol of substrate/mol enzyme/s. The reaction was strictly dependent on the presence of native enzyme, ring A, ring B, ATP, and divalent cations such as Mg²⁺ and Mn²⁺. EDTA (0.5 mM) reduced the enzyme activity to 1.4% of the maximal activity. Product formation in the novobiocic acid synthetase assay showed a linear dependence on the protein amount up to 5 μg of purified protein in the assay and on incubation time up to 10 min. The pH optimum was 8.0 in Tris-HCl buffer and 7.0 in phosphate buffer.

**Substrate Specificity**—Substrate specificity was tested with several analogues of ring A (Table III). Replacement of the dimethylallyl side chain of ring A by a geranyl side chain still allowed a substantial product formation (26% of the value obtained with the natural substrate). The geranylated reaction product was identified by EI-MS after preparative isolation. Some product formation was also detectable with 3-methyl-4-aminobenzoic acid. In contrast, 3-dimethylallyl-4-hydroxyxynamic acid was not accepted as a substrate, and neither were 4-hydroxybenzoic acid derivatives with bulky or polar substituents (Table III). No product formation was observed after incubation with 7-ami-no-4-methyl coumarin instead of ring B. Specificity for the nucleotide triphosphate was tested with 5 mM ATP, GTP, CTP, and TTP. Novobiocic acid synthetase activity was only detectable in the presence of ATP (100%) and to a much lesser extent with GTP (2.4%). The other nucleotides were completely ineffective. The reaction followed Michaelis-Menten kinetics for the substrates ring A and ring B in the presence of 5 mM ATP. Apparent Kₘ values of 19 μM for ring A and 131 μM for ring B were determined by the Lineweaver-Burk method.

**Mechanism of Activation**—Peptide synthetase and acyl-CoA ligase reactions usually proceed via acyl adenylates (32). Because the reaction ATP + carboxylic acid ⇌ acyl adenylate + PP, is an equilibrium reaction, the formation of [³²P]ATP can be observed when [³²P]PP is added to the reaction mixture. This ATP-PP exchange assay can be used to monitor the activity of adenylate forming enzymes. As shown in Table IV, the purified enzyme exhibited adenylate activity in this assay when incubated with ring A and ATP. No adenylate activity was observed when ring A was replaced by ring B. This strongly suggests that the reaction proceeds via the formation of a ring A-AMP intermediate followed by the transfer of the acyl group of ring A onto the amino group of ring B. An involvement of CoA seems unlikely, because the addition of CoA (0.5 mM) to the novobiocic acid synthetase assay did not increase novobiocic acid formation.

**DISCUSSION**

The antibiotic novobiocin contains two aromatic rings: ring A and ring B (Fig. 1). The linkage of those two rings by an ATP-dependent formation of an amide bond, i.e. the novobiocic acid synthetase reaction, has been demonstrated previously in crude enzyme extracts (19). Cloning of the novobiocin biosyn-

**TABLE II**

| Purification step               | Total protein | Total activity | Specific activity | Purification Yield % |
|---------------------------------|---------------|----------------|-------------------|----------------------|
| Crude extract                   | 289.9 mg      | 128.4 nkat      | 0.44 pkat/mg protein | 100                  |
| Nickel affinity chromatography  | 4.3 mg        | 69.3 nkat       | 16.1 pkat/mg protein | 36.6                 |
| Superdex™ 200 gel filtration    | 1.4 mg        | 70.8 nkat       | 50.6 pkat/mg protein | 115.0                |

*1 nkat was defined as the enzyme amount producing 1 nmol of novobiocic acid/s in the assay described under “Experimental Procedures.”*
TABLE III
Substrate specificity of novobiocic acid synthetase of S. spheroides NCIB 11891 after expression in E. coli XLI Blue MRF

| Substrates                  | Product formationa | Relative activity pmol s⁻¹ (mg protein)⁻¹ % |
|-----------------------------|--------------------|-----------------------------------------|
| Ring A and ring B           | 26,450             | 100                                     |
| Ring B and ring A analogues |                    |                                         |
| 3-Geranyl-4-hydroxybenzoic acid³ | 6.914              | 26.1                                    |
| 3,5-Di-3-tert-butyl-4-hydroxybenzoic acid³ | <0.014          | <0.001                                  |
| 3-Carboxymethylaminomethyl-4-hydroxybenzoic acid³ | <0.014          | <0.001                                  |
| 3-Methyl-4-hydroxybenzoic acid³ | <0.014              | <0.001                                  |
| 3-Methyl-4-aminobenzoic acid³ | 1.229              | 4.6                                     |
| 3-Cyclohexyl-4-hydroxybenzoic acid³ | <0.014          | <0.001                                  |
| 3-Dimethylallyl-4-hydroxyniminic acid³ | <0.014          | <0.001                                  |
| Ring A and ring B analogues |                    |                                         |
| 7-Amino-4-methyl coumarin   | <0.014             | <0.001                                  |
| L-Tyrosine                  | <0.014             | <0.001                                  |

³ Product formation with purified NovL was determined as described for the novobiocic acid synthetase assay under "Experimental Procedures," with 1 μl of the respective substrates. Data are the mean values of duplicate measurements.

a Values are the means of duplicate determinations.

Adenylation activity of the novobiocin acid synthetase

TABLE IV
Adenylation activity of the novobiocin acid synthetase

| Assay components | Product formationa |
|------------------|--------------------|
| Ring A, ATP, [⁴⁰⁰P]PP, NovL | 685.2 |
| Ring B, ATP, [⁴⁰⁰P]PP, NovL | 11.8 |
| ATP, [⁴⁰⁰P]PP, NovL | 12.4 |
| Ring A, ATP, [⁴⁰⁰P]PP, heat-denatured NovL | 3.2 |

a Values are the means of duplicate determinations.

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