Studies of Precursor-directed Biosynthesis with *Streptomyces* sp. Part 1. Isolation of Manumycin Analogues by Feeding of Aminobenzoic Acids as C_7N Starter Units

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Feeding experiments involving variants of the antibiotic manumycin (**1**) are described. Instead of the (unidentified) natural C_7N unit, isomeric aminobenzoic acids were used as alternative biosynthetic starting units. These were fed to *Streptomyces parvulus*, and manumycin-like metabolites were produced. Use of 3-aminobenzoic acid suppressed the biosynthesis of (**1**) and resulted in the synthesis of the manumycin analogue 64-*m*ABA (**2**), a new metabolite, the structure of which was determined. 4-Aminobenzoic acid induced the production of 64-*p*ABA (**4**), a metabolite without the chiral C_{13} side chain of (**1**); 2-aminobenzoic acid was not incorporated. The implications of these results for the biosynthesis of the natural C_7N starter unit, for the specificity of the involved enzymes, and for the possibility of using natural product variation to produce structurally varied antibiotics are discussed.

The antibiotics of the manumycin group, *e.g.* manumycin (**1**)[1,2] (the structure and absolute configuration of which have been described),[3,4] asukamycin,[5] U-62.162,[6] and U-56.407[7] are biosynthesized from structural elements in different biosynthetic pathways.[8-10] The multifunctional C_7N unit is of particular interest as it occurs in all manumycin-group antibiotics. Rickards' hyphothesis[11] that this unit may arise as a shunt metabolite from the shikimate pathway, predicting 3-aminobenzoic acid (ABA) and 3-amino-5-hydroxybenzoic acid (AHBA) as possible biosynthetic precursors, has been disproven.[9,10,12]

Further structural elements making up manumycin (**1**) are the two polyene chains, derived from the polyketide pathway,[10] and the 2-amino-3-hydroxycyclopent-2-enone moiety, formed from succinate and glycine *via* δ-aminolaevulinic acid (5-amino-4-oxopentanoic acid) as a biosynthetic intermediate.[8] Because the structural elements come from different biosynthetic pathways, one has the possibility of affecting manumycin biosynthesis by suitable modification of one or more of the precursor units. We have used *Streptomyces parvulus* (Tü 64) for our studies of precursor-directed biosynthesis. We observed that feeding of non-physiological amounts of artificial C_7N precursor during the stationary growing phase allowed us to override the biosynthesis or incorporation of the unidentified natural C_7N unit, the central manumycin starter unit. We hoped that such experiments would enable isolation of new manumycin derivatives and promote a better understanding of the biosynthesis of the manumycin-group antibiotics. In this paper we describe feeding experiments with isomeric aminobenzoic acids, and report the isolation and identification of two new natural manumycin analogues.

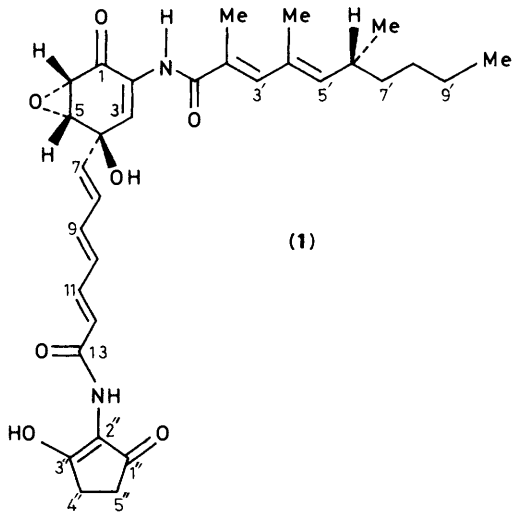
Feeding Experiments.—In separate experiments the isomeric aminobenzoic acids (55 mM) were fed to *Streptomyces parvulus* (strain Tü 64) about 40 h after inoculation during the stationary growing phase. The cultures (in shaking flasks or a 15 l fermentor) were harvested about 32 h later and the mycelium was extracted with acetone; the procedure was analogous to that for manumycin isolation.[3] The resulting crude products were examined by t.l.c. to obtain the metabolite pattern, which was compared with a control from an unfed fermentation. The Table shows the absence of manumycin in all feeding experiments, while the accompanying red C_{25} prodigiosins were produced in lower quantities, indicating that absolute suppression of normal secondary metabolism did not occur. Feeding of 3-aminobenzoic acid resulted in a new yellow metabolite, 64-*m*ABA, detectable on t.l.c. [R_F 0.60 in $CHCl_3$–MeOH (9:1 v/v); *cf.* (**1**) R_F 0.51]. Feeding of 4-aminobenzoic acid led to the biosynthesis of the yellow 64-*p*ABA (R_F 0.42); use of 2-aminobenzoic acid did not result in a new product. The feeding experiments were optimized in the case of 3-aminobenzoic acid by determination of the yield of 64-*m*ABA as a function of fed amino acid (Figure 1). At 7 mM 3-aminobenzoic acid drastically suppressed the biosynthesis of manumycin (**1**), but no 64-*m*ABA was detected. At 55 mM highest yields of (**2**) were obtained whereas at 2—4 mM there was no effect on normal manumycin production.[12]

Identification of 64-*m*ABA (**2**).—The crude extract of the mycelium from a feeding experiment with 3-aminobenzoic acid was purified by column chromatography on Sephadex LH-20 and silica gel RP-8 giving the pure 64-*m*ABA (**2**), which is soluble in methanol, acetone, and chloroform and insoluble in water and hexane. The yields of (**2**) varied between 40 and 123 mg per litre of culture broth in different fermentations, and its spectroscopic data [*e.g.* ^1H n.m.r. (Figure 2) and ^{13}C n.m.r. spectra] showed a close similarity to those of manumycin (**1**)[3] and its aromatic derivatives deoxymanumycin and dideoxymanu-

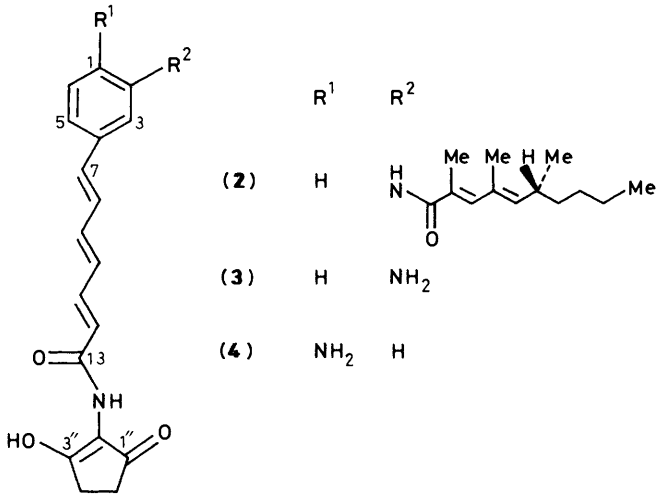
Table. Feeding of isomeric aminobenzoic acids to *Streptomyces parvulus* (strain Tü 64)

Precursor (mM)	Manumycin (**1**)	Production of prodigiosins[a]	New products
Control	*ca.* 100 mg l^{-1}	Strong	
2-Aminobenzoic acid (*o*ABA)(54.7)		Weak	
3-Aminobenzoic acid (*m*ABA)(54.7)		Weak	64-*m*ABA (**2**) (123 mg l^{-1})
4-Aminobenzoic acid (*p*ABA)(54.7)		Strong	64-*p*ABA (**4**) (80 mg l^{-1})

[a] By observing the colour of the mycelium and t.l.c. control of the crude products.



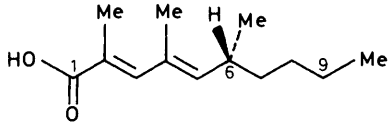
(1)



(2) R¹ = H, R² = Me Me H Me Me

(3) R¹ = H, R² = NH₂

(4) R¹ = NH₂, R² = H



(5)

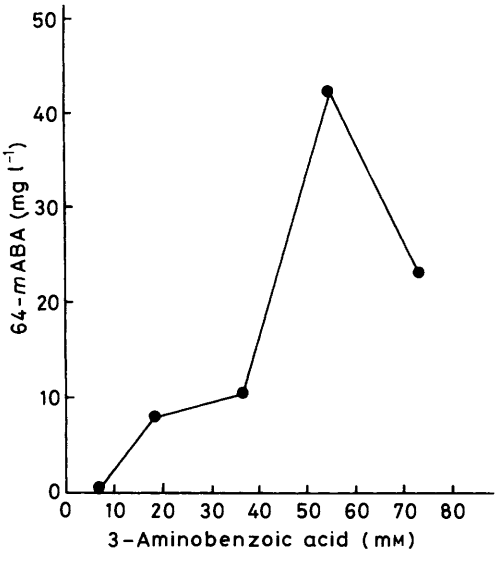


Figure 1. Yield of 64-*m*ABA (**2**) as a function of fed 3-aminobenzoic acid

mycin.[13] On the basis of these data and the high resolution mass spectrum [*m/z* 502 (M^+, $C_{31}H_{38}N_2O_4$)] the structure (**2**) was confirmed. N.m.r. correlation spectroscopy (^1H–^1H and ^1H–^{13}C; see Figure 3) enabled the signals to be assigned,* and indicated the presence of the 3-aminobenzoic acid precursor without further substitution of the aromatic nucleus. The remaining structural elements are identical with those of manumycin. The ^3J values for 8-H, 10-H, and 12-H indicated the all-*trans* configuration of the triene chain.

Hydrolysis of (**2**) with methanolic KOH under conditions more drastic than those necessary for manumycin (**1**)[3] liberated two main products, which could be separated on silica gel and Sephadex LH-20 columns in chloroform–methanol systems. The first was identified as the 2,4,6-trimethyldecadienoic acid (**5**) from its physicochemical data [identical with those of the same acid derived from (**1**)[3]]. The agreement of the optical rotation values from the two side-chain acids established the absolute configuration of 64-*m*ABA (**2**) as 6′*R*. The second compound isolated was a yellow amorphous powder. N.m.r. spectra indicated the lack of the C_{13} side chain and an unchanged aromatic nucleus, triene chain, and C_5N moiety. Because the n.m.r. spectra are so complex in the aromatic/olefinic region, all signals have not yet been assigned. The high resulution mass spectrum, with the molecular ion at *m/z* 310 gave the molecular formula ($C_{18}H_{18}N_2O_3$) and indicated structure (**3**) for this hydrolysis product.

To establish whether 3-aminobenzoic acid is the direct precursor of the C_7N unit in 64-*m*ABA (**2**), 3-aminobenzoic [^{13}C]-acid (99% enriched; 500 mg l^{-1}) mixed with unlabelled acid (5 g l^{-1}) was fed to *Streptomyces parvulus*. The ^{13}C n.m.r. spectrum showed a 10-fold increase of the signal at δ 136.9, which had been assigned to C-7. Thus, C-7 in (**2**) arises directly from the carbonyl group of 3-aminobenzoic acid. Remarkably, 3-aminobenzoic [^{13}C]acid fed in amounts which did not suppress manumycin production was not incorporated into the parent manumycin (**1**).[12]

Identification of 64-*p*ABA (**4**).—4-Aminobenzoic acid fed during the secondary growing phase of *Streptomyces parvulus* resulted in a new yellow compound, which was chromatographically purified and characterized by its spectroscopic data. These data were similar to those of (**3**), which possessed the same molecular formula. The fact that the triene side chain was positioned *para* to the amino group was shown by both, the ^{13}C n.m.r. spectrum (with identical chemical shifts for the C-2/-6 and C-3/-5 exhibiting twice the intensity of the other signals) and the ^1H n.m.r. spectrum [with *ortho*-coupled hydrogen atoms at δ 6.57 (*J* 8.5 Hz, 2- and 6-H) and 7.23 (*J* 8.5 Hz, 3- and 5-H)]. By

* The numbering system for structures (**2**)—(**4**) corresponds to that used for the parent manumycin.

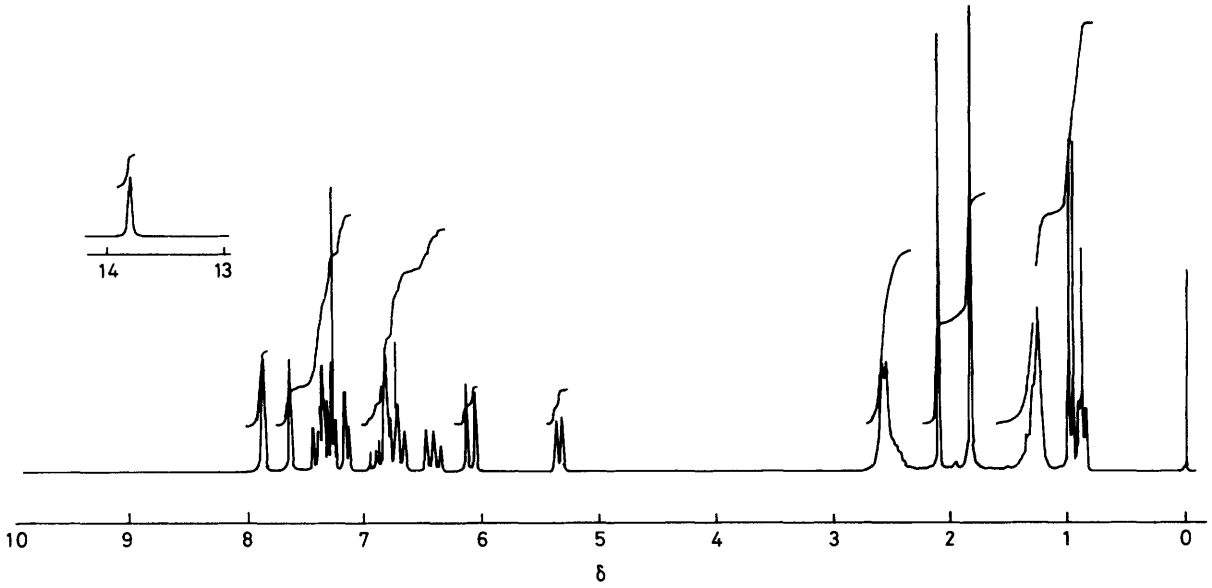


Figure 2. ^{1}H N.m.r. spectrum of 64-*m*ABA (**2**) in $CDCl_3$ at 200 MHz

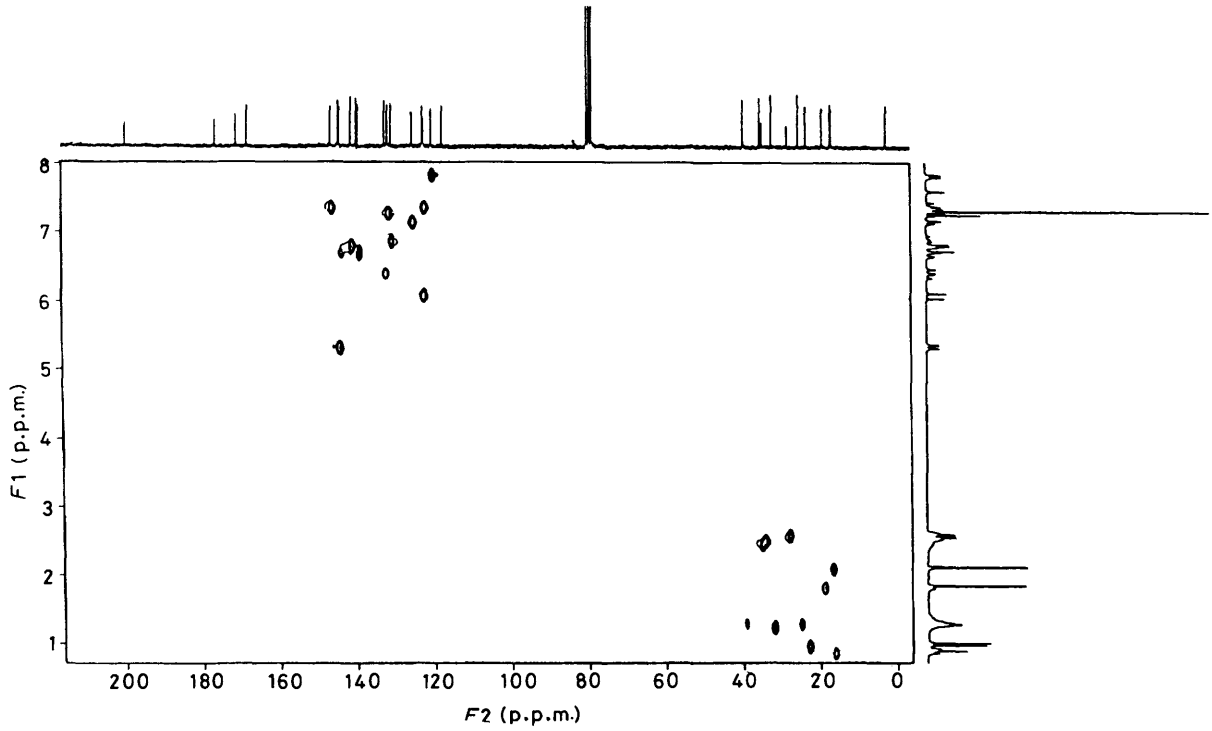


Figure 3. ^{1}H–^{13}C COSY n.m.r. spectrum of 64-*m*ABA (**2**) in $CDCl_3$

analogy with (**1**) and (**2**), we propose that the configuration of the triene chain is all-*trans*. Thus, the structure of 64-*p*ABA is (**4**).

Discussion.—The feeding experiments described here offer insights into the structural behaviour of the C_7N precursor in manumycin biosynthesis. The natural multifunctional C_7N unit leading to (**1**) could be replaced by 3-aminobenzoic acid to yield 64-*m*ABA (**2**) in amounts which correspond to normal manumycin production. The *meta*-relationship of the amino and carboxy groups is of fundamental importance for the binding of this biosynthetic unit to the polyene chains. In the case of 4-aminobenzoic acid [leading to (**4**)], the precursor is extended only at the carboxy group. Additionally, this experiment offers evidence regarding the sequence of biosynthesis and chain extension. The lack of incorporation of 2-aminobenzoic acid may be a result of steric hindrance by the *ortho*-substituent. The intact incorporation of 3-aminobenzoic

[^{13}C]acid into 64-*m*ABA (**2**) without further functionalisation indicates that the C_7N starter unit in the biosynthesis of manumycin (**1**) cannot be *m*ABA. In accord with the results of other feeding experiments,[12] we assume that the natural precursor is non-aromatic.

The enzyme recognizing the C_7N starter unit, which may be activated by formation of a coenzyme A derivative followed by C–C linkage with malonyl-coenzyme A *via* polyketide biosynthesis, must possess low specifity, because it accepts aromatic amino acids. This contrasts with the observation that no minor compounds similar to manumycin were isolated under normal fermentation conditions; this predicts a highly specific manumycin synthetase system. The non-physiological amounts of aromatic amino acids used may overcome the specificity of the enzymes involved. We assume that the natural C_7N unit has free carboxy and amino groups in a *meta*-orientation with distances similar to those in ABA. Perhaps this is the only requirement for the biosynthesis of manumycin or manumycin analogues with both polyene chains. We cannot decide whether 3-aminobenzoic acid suppresses the biosynthesis of the natural C_7N unit (perhaps *via* feedback inhibition) or competes for the enzymes involved because of its higher concentration. In the latter case we might expect the unidentified natural C_7N precursor of manumycin to be enriched in the culture broth and probably to be isolable. The fact that small amounts of *m*ABA (7 mM) suppress manumycin biosynthesis without producing 64-*m*ABA (**2**) provides evidence of an inhibition process which may affect one or more of the steps on the pathway to the natural C_7N unit.

Previous studies to replace the natural C_7N unit in ansamitocin,[14] rifamycin,[15] and actamycin[16] by directed biosynthesis using aromatic amino acids were not successful. The C_7N unit in these antibiotics were shown to be biosynthesized from shikimate pathway intermediates *via* 3-amino-5-hydroxybenzoic acid (AHBA), in contrast to manumycin.[10,12] Feeding of high concentrations of artificial precursor as a means of overriding the normal biosynthesis of manumycin may well be a new approach to the biosynthetic production of structurally modified antibiotics.

The production of new secondary metabolites *via* precursor-directed biosynthesis is an attractive and simple method[17] requiring no blocked mutants of the antibiotic-producing strain as in the case of mutasynthesis[18–20] (used to create new aminocyclitols,[21] macrolides,[22] or anthracyclines).[23] In most of the descriptions of new antibiotics derived by directed biosynthesis, the variations of the parent compounds were initiated only at a late stage of the biosynthetic pathway and their formation can be put down to the lack of specificity of the enzymes involved (mostly amidases and esterases). Examples are the penicillins,[24] celesticetin,[25,26], the echinomycins,[27,28], paulomycin,[29,30] and saframycin.[31] In the mitomycins[32] and nikkomycin,[33] the basic skeleton of the antibiotic has been shown to be modified by precursor-directed biosynthesis. In most of the cases described the lack of specificity of the antibiotic synthetase enzymes is indicated by the discovery of analogues in unfed fermentations. Since there were no manumycin analogues detectable in normal fermentations, our concept of directed biosynthesis differs markedly from others which have been described. We fed non-physiological amounts of a similar starter unit, which became a central part of the antibiotic molecule by formation of a C–C linkage: normal biosynthesis was overridden. The artificial starter unit suppresses the incorporation or biosynthesis of the natural one, resulting in a lack of manumycin production, but does not affect the following biosynthetic steps. Possibilities of replacing a starter unit were previously noted in the cases of pyrrolnitrin[34,35] and indolmycins[36] synthesized from tryptophan analogues, but these analogues did not suppress the production of the parent antibiotic totally.

Experimental

M.p.s were determined with a Reichert hot-stage microscope. I.r. spectra (KBr discs) were recorded with a Perkin-Elmer 297 spectrometer and u.v. spectra with a Kontron Uvikon 860 spectrometer. Optical rotations were recorded with a Perkin-Elmer 241 polarimeter. The intensities of t.l.c. spots were evaluated with a Desaga CD 50 Chromatogram Densitometer. N.m.r. spectra were determined with a Varian XL-200 or Varian VXR-200 spectrometer (^{1}H at 200 MHz; ^{13}C at 50.3 MHz); chemical shifts are expressed as δ values (p.p.m.) with Me_4Si as internal standard. The multiplicities of the ^{13}C signals were assigned by using APT or DEPT techniques. The mass spectra were taken with a Varian MAT-311a (electron impact) mass spectrometer at 70 eV.

T.l.c. was performed on silica gel plates (Macherey & Nagel SIL G/UV 254 + 366; 0.25 mm; silica gel on glass), and column chromatography on silica gel 60 (0.08 mm; Macherey & Nagel), silica gel RP-8 (Lobar Fertigsäule; 40—63 μm; Merck), or Sephadex LH-20 (Pharmacia).

Fermentation and Feeding Experiments.—The fermentation conditions for *Streptomyces parvulus* (strain Tü 64) have been described in detail.[3] Feeding experiments with various unlabelled precursors were carried out using 100 ml of culture medium (2% mannitol; 2% degreased soybean meal) in 1000 ml Erlenmeyer flasks which were shaken for 72 h; 36—40 h after inoculation the precursor, dissolved in a small amount of sterile water and adjusted to pH 7.0 with 2M NaOH, was added under sterile conditions (see Table). The cultures were harvested as described for manumycin[3] and the crude products were screened by t.l.c. in various solvent systems with use of various staining reagents.

N-(2-Hydroxy-5-oxocyclopent-1-enyl)-7-[3-(2,4,6-trimethyldeca-2,4-dienoylamino)phenyl]hepta-2,4,6-trienamide (64-*m*ABA) (**2**).—After 36 h fermentation in a 15 l fermentor, 3-aminobenzoic acid (55 mM), dissolved in a small amount of sterile water and adjusted to pH 7.0, was fed to the Tü 64 culture. After 74 h the mycelium was extracted and defatted as described for manumycin[3] to yield a dark brown amorphous powder. The crude product was chromatographed twice on Sephadex LH-20 columns (100 × 2.5 cm; $CHCl_3$) and was further purified on an RP-8 silica gel column [acetonitrile–water (3:1 v/v)] to yield, in an optimal fermentation, the pure yellow amorphous *metabolite* 64-mABA (**2**) (1.845 g; 123 mg per litre of culture broth), m.p. 196—197 °C; $[\alpha]_D^{22}$ − 213° (*c* 0.15 in $CHCl_3$); $\nu_{max.}$(KBr) 3 420, 3 260, 2 940, 2 910, 1 605, 1 575 sh, 1 530, and 996 cm^{-1}; $\lambda_{max.}$(MeOH) 351 (ε 40 800) and 263 nm (25 100); δ_C($CDCl_3$) 197.4 (s, C-1″), 174.3 (s, C-3″), 168.7 (s, C-1′), 165.9 (s, C-13), 144.0 (d, C-11), 142.1 (d, C-5′), 141.6 (d, C-9), 138.9 (d, C-3′), 138.8 (s, C-2), 137.4 (s, C-4), 136.9 (d, C-7), 130.2 (d, C-10), 130.1 (s, C-2′), 129.4 (s, C-4′), 129.3 (d, C-6), 128.5 (d, C-8), 123.0 (d, C-5), 120.4 (d, C-12), 120.1 (d, C-1), 118.0 (d, C-3), 115.3 (s, C-2″), 37.1 (t, C-7′), 32.8 (d, C-6′), 32.2 (t, C-5″), 29.8 (t, C-8′), 25.7 (t, C-4″), 22.8 (t, C-9′), 20.8 (q, C-13′), 16.6 (q, C-12′), 14.4 (q, C-10′), and 14.1 (q, C-11′); δ_H($CDCl_3$; see Figure 2) 0.86 (t, *J* 6.4 Hz, 10′-H_3), 0.93 (d, *J* 6.4 Hz, 13′-H_3), 1.14—1.42 (br m, 7′-, 8′-, and 9′-H_2), 1.84 (d, *J* 1.6 Hz, 12′-H_3), 2.12 (d, *J* 1.6 Hz, 11′-H_3), 2.34—2.50 (br m, 6′-H), 2.58—2.60 (br s, 4″- and 5″-H_2), 5.36 (d, *J* 10 Hz, 5′-H), 6.09 (d, *J* 15 Hz, 12-H), 6.42 (dd, *J* 13.5 and 12 Hz, 10-H), 6.62—6.88 (m, 7- and 9-H) [including 6.82 (3′-H)], 6.90 (dd, *J* 15.5 and 11 Hz, 8-H), 7.15 (br d, *J* 7.5 Hz, 5-H), 7.22—7.46 (m, 1-, 6-, and 11-H), 7.64 (br s, NH), 7.86 (br s, 3-H), 7.88 (br s, NH), and 13.80 (br s, OH); *m/z* 502.2832 (21%, M^+; $C_{31}H_{38}N_2O_4$), 417 (24), 324 (11), 193 (38), 123 (11), and 109 (64) (Found: C, 74.0; H, 7.8; N, 5.5. $C_{31}H_{38}N_2O_4$ requires C, 74.1; H, 7.6; N, 5.6%).

Hydrolysis of 64-mABA (**2**).—Compound (**2**) (94 mg) dissolved in methanolic 20% KOH (50 g) was refluxed for 24 h. The yellow mixture was adjusted to pH 2 with aqueous oxalic acid and extracted with $CHCl_3$ (2 × 300 ml). The combined organic layers were dried (Na_2SO_4) and evaporated to obtain a yellow amorphous powder. The crude product was chromatographed on a silica gel column [45 × 2.5 cm; $CHCl_3$—MeOH (9:1 v/v)] to yield three main products: (a) (**2**) (5.8 mg), R_F 0.60; (b) a pale yellow oil (30.0 mg), R_F 0.43; and (c) a yellow powder (34.2 mg), R_F 0.39.

Fraction (b) was further purified on a Sephadex LH-20 column (45 × 2.5 cm; $CHCl_3$) to yield 2,4,6-trimethyldeca-2,4-dienoic acid (**5**) (20.0 mg, 54%), with spectroscopic data as described in ref. 3.

Fraction (c) was chromatographed on a Sephadex LH-20 column (50 × 2.5 cm; $CHCl_3$) to yield N-(1-*hydroxy*-5-*oxocyclopent*-1-*enyl*)-7-(3-*aminophenyl*)*hepta*-2,4,6-*trienamide* (**3**) as a yellow amorphous powder (30.2 mg, 55%), m.p. 256 °C; $\nu_{max.}$(KBr) 3 440, 3 380, 3 260, 1 680sh, 1 610, 1 550, and 1 008 cm^{-1}; $\lambda_{max.}$(MeOH) 342 (ε 44 500) and 259 nm (24 600); δ_H[$(CD_3)_2SO$] 2.09 (s, 4″-H_2), 2.48 (s, 5″-H_2), 3.30 (br s, NH, overlapped by HOD), 6.43—7.08 (9 H, m), 7.29 (dd, *J* 15.0 and 12.5 Hz, 11-H), and 9.91 (br s, OH); δ_C [$(CD_3)_2SO$] 166.1 (s, C-13), 148.8 (s, C-2), 142.5 (d, C-11), 137.3 (d, C-9), 136.9 (d, C-7), 129.9 (s, C-4), 114.8 (s, C-2″), and 28.8 (br t, C-4″ and -5″) [the signals at 129.1 (d), 127.4 (d), 121.2 (d), 119.0 (d), 114.9 (d), 114.6 (d) and 112.5 (d) could not be assigned definitely to C-1, C-3, C-5, C-6, C-8, C-10, and C-12; the signals for C-1″ and C-3″ were not observed at 50.3 MHz]; *m/z* 310.1317 (13%, M^+; $C_{18}H_{18}N_2O_3$), 198 (49), 180 (15), 170 (92), and 132 (100).

Feeding Experiment with 3-*Aminobenzoic* [^{13}C]*Acid*.—3-Aminobenzoic [^{13}C]acid (99% enriched; 200 mg) and unlabelled 3-aminobenzoic acid (2 g) (both adjusted to pH 7.0 with 2M NaOH) were added to a 40 h-old culture of Tü 64 (4 × 100 ml culture medium in 1 000 ml Erlenmeyer flasks, shaken at 28 °C). The cultures were harvested 74 h after inoculation; extraction and purification were performed as before to yield 64-*m*ABA (**2**) (11.2 mg).

N-(1-*Hydroxy*-5-*oxocyclopent*-1-*enyl*)-7-(4-*aminophenyl*)-*hepta*-2,4,6-*trienamide* (64-pABA) (**4**).—4-Aminobenzoic acid (55 mM) was fed to a 36 h-old culture (10 × 100 ml culture medium in 1 000 ml Erlenmeyer flasks shaken at 28 °C) of strain Tü 64. After 74 h the mycelium was separated and extracted as for manumycin.[3] The dark brown crude product was chromatographed twice on a silica gel column [30 × 5 cm; $CHCl_3$–MeOH (9:1 v/v)] to yield the product (**4**) (80.4 mg l^{-1}) as a yellow amorphous *powder*, m.p. 242 °C; $\nu_{max.}$(KBr) 3 440, 3 350, 3 240, 2 930, 2 860, 1 710sh, 1 615sh, 1 585, and 1 000 cm^{-1}; $\lambda_{max.}$(MeOH) 392 (ε 31 500), 261 (14 400), and 201 nm (15 200); δ_C[$(CD_3)_2SO$] 166.4 (s, C-13), 149.6 (s, C-1), 143.1 (d, C-11), 142.5 (d, C-9), 137.8 (d, C-7), 128.3 (d, C-3 and C-5), 127.3 (d, C-10), 124.1 (s, C-4), 122.9 (d, C-8), 119.4 (d, C-12), 114.9 (s, C-2″), 113.8 (d, C-2 and C-6), and 28.8 (br t, C-4″ and -5″) (the signals for C-1″ and C-3″ were not observable at 50.3 MHz); δ_H [$(CD_3)_2SO$] 2.10 (s, 4″-H_2), 2.47 (s, 5″-H_2), 3.30 (br s, NH, overlapped by HOD), 5.50 (br s, NH), 6.34—6.94 (7 H, m), [including 6.57 (d, *J* 8.5 Hz, 2- and 6-H)], 7.23 (d, *J* 8.5 Hz, 3- and 5-H), 7.30 (dd, *J* 14.5 and 12 Hz, 11-H), and 9.94 (br s, OH); *m/z* 310.1317 (8.4%, M^+; $C_{18}H_{18}N_2O_3$), 198 (10.4), 170 (40.7), and 132 (100).

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