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Molecular Analysis of the Benastatin Biosynthetic Pathway and Genetic Engineering of Altered Fatty Acid−Polyketide Hybrids

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Abstract: The entire gene locus encoding the biosynthesis of the potent glutathione-*S*-transferase inhibitors and apoptosis inducers benastatin A and B has been cloned and sequenced. The cluster identity was unequivocally proven by deletion of flanking regions and heterologous expression in *S. albus* and *S. lividans*. Inactivation and complementation experiments revealed that a KSIII component (BenQ) similar to FabH is crucial for providing and selecting the rare hexanoate PKS starter unit. In the absence of BenQ, several novel penta- and hexacyclic benastatin derivatives with antiproliferative activities are formed. In total, five new compounds were isolated and fully characterized, and the chemical analysis was confirmed by derivatization. The most intriguing observation is that the *ben* PKS can utilize typical straight and branched fatty acid synthase primers. If shorter straight-chain starters are utilized, the length of the polyketide backbone is increased, resulting in the formation of an extended, hexacyclic ring system reminiscent of proposed intermediates in the griseorhodin and fredericamycin pathways. Analysis and manipulation of the hybrid fatty acid polyketide pathway provides strong support for the hypothesis that the number of chain elongations is dependent on the total size of the polyketide chain that is accommodated in the PKS enzyme cavity. Our results also further substantiate the potential of metabolic engineering toward polyphenols with altered substituents and ring systems.

Introduction

Aromatic polyketides formed by type II polyketide synthases (PKS) comprise an important and structurally diverse group of bacterial secondary metabolites.[1−3] Many of these compounds that are produced by soil-borne and marine Gram-positive Actinomycetes have emerged as clinically useful drugs or drug leads, such as the tetracyclines and anthracyclines.[4] With the advent of molecular tools and recombinant methods applicable to Actinomycetes,[5,6] it has become feasible to investigate bacterial aromatic polyketide biosynthesis at the genetic, biochemical, and structural levels,[7−10] which has eventually set the basis for engineering novel natural product derivatives.[11−14]

Bacterial type II PKS are typically composed of a single set of iteratively used individual proteins.[2,3,8] A hallmark of type II PKS is the so-called minimal PKS composed of a β-ketoacyl synthase (KS_α)/chain length factor (CLF, or KS_β) heterodimer, and an acyl carrier protein (ACP). The vast diversity of aromatic polyketides is first a function of the chain length of the polyketide backbone, and the size and topology of the ring system (linear, angular, or discoid).[15] These factors are controlled by the minimal PKS components, as well as additional keto reductases and cyclases. Polyketide tailoring events, such as oxygenation, halogenation, acylation, alkylation, and glycosyl transfer, introduce another level of diversity.[11] It should be highlighted that unusual ring substituents may also result from PKS priming with alternate PKS starter units, which can significantly influence the bioactivity profile of the natural product.[16]

The pradimicin-type metabolites, which share a common benzo[*a*]naphthacene scaffold, have attracted considerable interest in the medical,[17,18] microbial,[19,20] and chemical communities[21−25] due to their various biological activities.

The pradimicins and benanomicins isolated from *Actinomadura* sp. are well-known potent antimycotics, and a synthetic derivative has entered clinical trials in the 1990s. The structurally

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related *Streptomyces* metabolites benastatin A and B (**1**, **2**) are active against Gram-positive bacteria including methicillin-resistant strains. Furthermore, they were identified as potent inhibitors of glutathione *S*-transferase (GST).[26] GSTs catalyze the conjugation of glutathione to the electrophilic center of various carcinogens and mutagens, and trigger the most important phase II detoxification pathway, which protects animal cells against the toxic and neoplastic effects of the carcinogens, electrophiles, and products of oxidative stress.[27,28] GSTs are regarded as attractive drug targets because their inhibitors, such as ethacrynic acid, can induce apoptosis in the Jurkat human T cell line[29] and other cell lines,[30] and GSTs expressed in resistant cancer cells are likely involved in the detoxification of anti-cancer drugs and prevention of apoptosis.[31] Interestingly, benastatin A is capable of inducing apoptosis and cell cycle arrest in colon 26 cells, albeit this effect is an unlikely direct result of GST inhibition.[31]

Aside from the intriguing biological activities, the benastatins have peculiar structural characteristics. In contrast to the pradimicins, they feature an unusual pentyl side chain in place of a methyl group in the pradimicin structure, which points toward a hexanoate biosynthetic building block.[16] Labeling experiments by Aoyama et al. revealed that the benastatins are composed of 14 acetate units and two *S*-adenosyl methionine (SAM)-derived methyl groups. Thus, the benastatins rank among polyphenols with the longest polyketide backbone.[32] In addition, instead of the typical benzo[*a*]naphthacene quinone chromophore, as in the related bequinostatins (Figure 1),[33,34] the benastatins bear a highly unusual geminal dimethyl ring substitution. Similar moieties on polyphenols are only known from resistomycin[35] and antibiotic L-755,805.[36]

Here, we report the cloning, sequencing, and heterologous expression of the entire benastatin biosynthesis gene cluster and provide an insight into the biosynthesis of this unusual polyketide−fatty acid hybrid. In addition, we succeeded in efficiently manipulating the pathway, which led to the formation of benastatin analogues with altered side chains and ring systems.

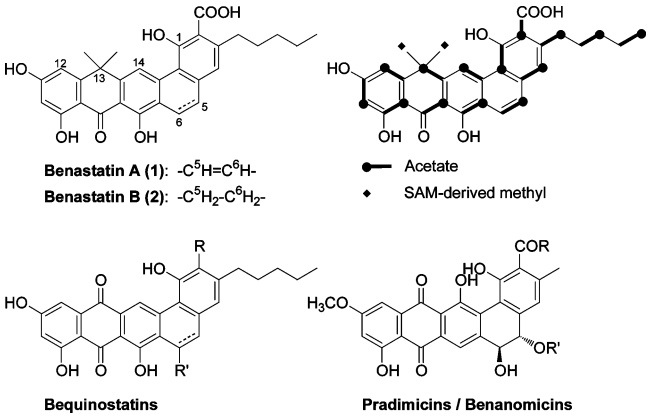

Figure 1. Structures of the benastatins and related bequinostatins, pradimicins, and benanomicins and ^{13}C isotope labeling pattern of **1**.

Results and Discussion

Identification, Cloning, and Heterologous Expression of the Benastatin Biosynthetic Gene Cluster. To localize and clone the benastatin biosynthesis genes, a cosmid library was constructed from genomic DNA of benastatin producer *Streptomyces* sp. A2991200 using *E. coli*−*Streptomyces* shuttle cosmic vector pKJ01.[15] Because the structures of the benastatins suggest a biosynthetic route similar to those of pradimicin or griseorhodin, the 1920-clone library was screened with a heterologous KS_α gene probe from the griseorhodin (*grh*) biosynthesis gene cluster kindly provided by J. Piel.[37] All positive clones (38) were subjected to restriction mapping and further Southern hybridization and shuttled into *Streptomyces* expression hosts. Introduction of cosmid p5H09 into *Streptomyces lividans* TK23 by PEG-mediated protoplast transformation conferred to the host the ability of producing benastatins. Polyketide production was clearly detectable in an extract of a *S. lividans* TK23/p5H09 culture by the characteristic orange fluorescence of the polyphenols on TLC plates. The identity of benastatins produced by the recombinant strain was further established by HPLC and MS compared to the wild-type strain. We noted that in an upscaled fermentation with increased aeration both wild-type and the transformed host also produce the 13-hydroxy-norbenastatin derivatives BE 43767A (**3**) and TAN 1532B (**4**), which have been described in the patent literature only (Figure 5).[38,39]

Because cosmid p5H09 apparently contains all genes necessary to encode benastatin biosynthesis, the entire cosmid (∼42 kb) was subjected to shotgun sequencing. The gene locus encoding benastatin biosynthesis was narrowed down to ∼17 kb by excision of nonessential flanking regions by PCR-targeted recombination,[40,41] yielding cosmid pXU130 that contains all

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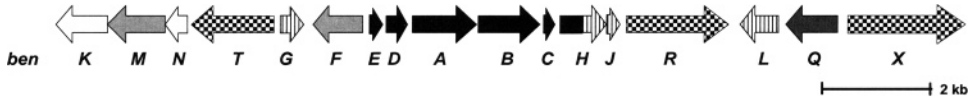

Figure 2. Organization of the benastatin biosynthesis gene cluster in *Streptomyces* sp. A2991200. Each arrow indicates the direction of transcription and relative sizes of the ORFs deduced from analysis of the nucleotide sequence. Black ORFs, minimal PKS and cyclase/aromatase genes; dark gray ORF, KSIII gene; hatched ORFs, putative oxidoreductase genes; light gray ORFs, SAM synthetase and methyl transferase genes; checked ORFs, probable regulatory and resistance genes; white ORFs, genes of unidentified function.

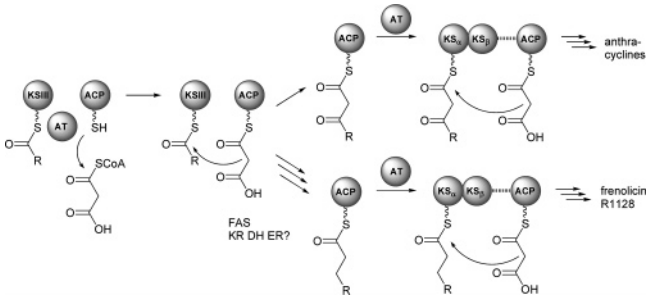

Figure 3. PKS priming with non-acetate starter units involving a KSIII.

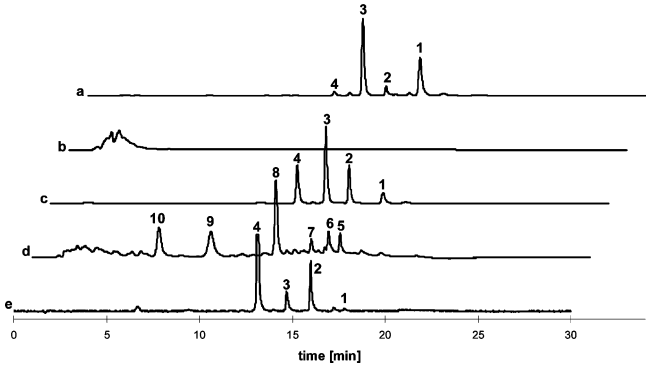

Figure 4. HPLC profiles of extracts from wild type (a), host *S. albus* (b), *S. albus*/pXU130 expressing essential pathway genes (c), *S. albus*/pXU132 lacking the *benQ* gene (d), and complemented mutant *S. albus*/pXU132+pXU110 (e). Production levels are at the same order of magnitude.

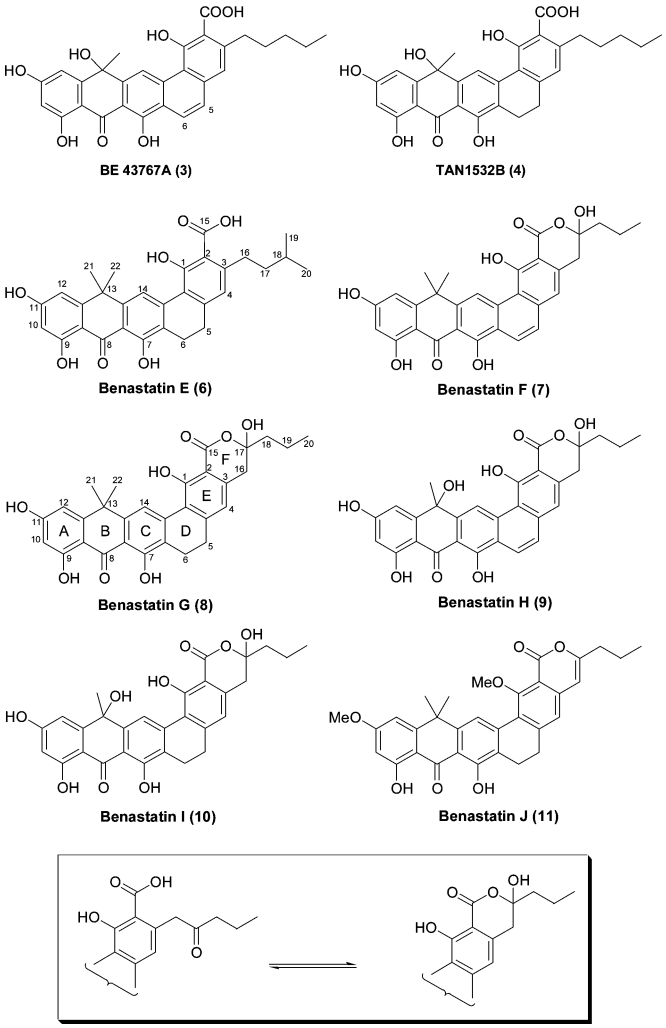

Figure 5. Structures of novel benastatin derivatives (the numbering scheme follows that of original benastatins).

essential *ben* biosynthesis genes. Frame analysis revealed 17 ORFs, 14 probable structural genes, and three regulatory and resistance genes that were identified on the basis of homology searches. The organization of the *ben* gene cluster is graphically presented in Figure 2, and the results of the sequence analysis are summarized in Table 1. The putative functions of deduced gene products were assigned by sequence comparisons with database proteins.

Enzymes Involved in the Formation of the Pentacyclic Ring System. The minimal PKS, which is responsible for the assembly of the benastatin poly-β-keto chain, is encoded by *benA-C*. All deduced gene products are closely related to known aromatic PKS that are involved in the biosynthesis of long-chain polyketides, such as fredericamycin (*fdm*),[42] rubromycin (*rub*),[43] and griseorhodin (*grh*).[37]

The minimal *ben* PKS genes are flanked by three probable cyclase genes, *benE*, *benD*, and *benH*, which are implicated in the formation of the pentacyclic ring system. The deduced protein of *benH* is bifunctional, as it is composed of cyclase (pfam03364) and monooxygenase (pfam03992) domains. A similar architecture has been reported for RubF, GrhT, and TcmN, all of which are bifunctional proteins with the same type of polyketide cyclase domain at the N-terminus, whereas the C-terminal parts are variable (e.g., the C-terminus of both RubF and GrhT is a short-chain dehydrogenase,[37] while TcmN bears an *O*-methyltransferase domain.)[44] The conserved N-terminal cyclase/dehydrase domain might be responsible for initial folding of the nascent linear polyketide intermediate and subsequent cyclization between C9 and C14 (Figure 6). BenE is another probable cyclase that is strikingly similar to GrhQ and FdmD from the *grh* and *fdm* pathways. In addition, BenE has high similarity/identity scores (70%/53%) with TcmI,[45] which is supposedly involved in the cyclization of the tetracenomycin D ring.[46] The gene product of *benD* is closely related to

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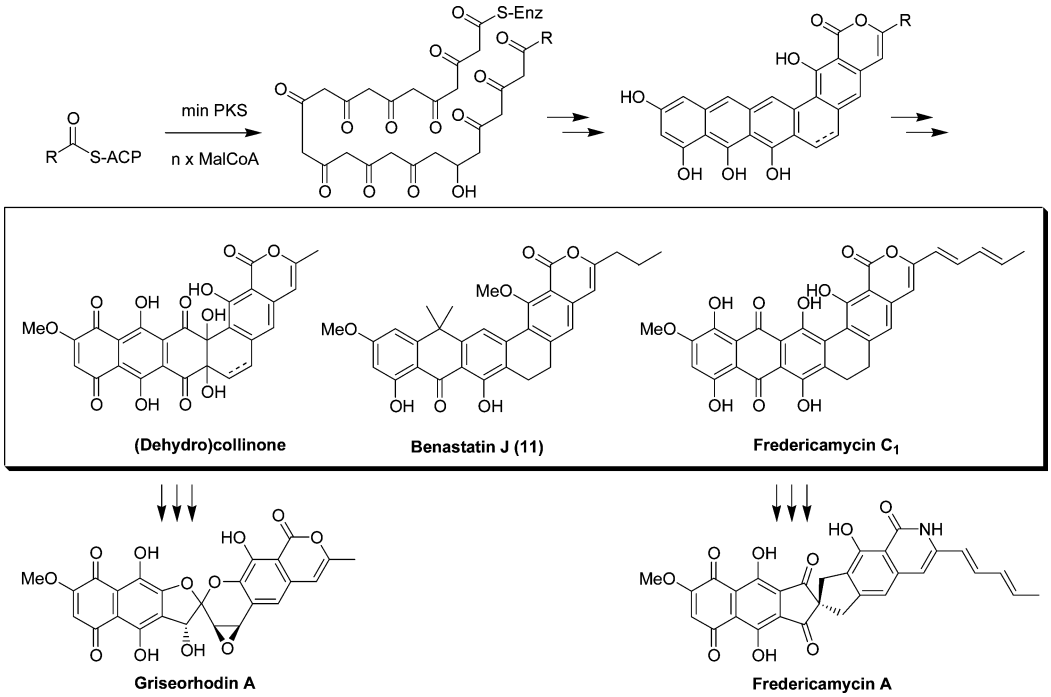

Figure 6. Priming of the *ben* PKS by linear and branched short-chain fatty acids (R = CH_3 or OH).

Table 1. Proposed Functions of the Deduced Gene Products Encoded by the *ben* Biosynthetic Gene Cluster

deduced protein	size (aa)	proposed function	closest relative (protein, origin)	similarity/ identity, %	accesssion number
BenK	322	carbohydrate kinase	*Thermobifida fusca* YX	72/59	YP_289073
BenM	397	*S*-adenosyl methionine synthetase	MetK, *Streptomyces fradiae*	86/81	AAK98791
BenN	129	shikimate 5-dehydrogenase	*Polaromonas* sp. JS666	48/32	ZP_00506655
BenT	512	transmembrane efflux protein	*Streptomyces avermitilis* MA-4680	73/60	BAC71669
BenG	151	unknown (monooxygenase)	RubQ, *Streptomyces collinus*	49/34	AAM97373
BenF	392	methyl transferase	*Streptomyces coelicolor* A3(2)	67/49	CAB76315
BenE	106	cyclase I	RubE, *Streptomyces collinus*	76/64	AAG03065
BenD	147	cyclase II	ORF II, *Streptomyces coelicolor*	71/54	CAA39407
BenA	422	ketosynthase (KSa)	RubA, *Streptomyces collinus*	80/68	AAG03067
BenB	409	chain-length factor (CLF, KSb)	FdmG, *Streptomyces griseus*	72/60	AAQ08917
BenC	86	acyl carrier protein	ORF 3, *Actinomadura hibisca*	64/46	BAA23146
BenH	279	bifunctional cyclase-monooxygenase	RubF, *Streptomyces collinus*/ ORF 8 *Actinomadura hibisca*	72/61 69/58	BAA23151 AAG03070
BenJ	115	monooxygenase	ORF 9, *Actinomadura hibisca*	61/50	BAA23152
BenR	656	transcriptional activator	Snora, *Streptomyces nogalater*	52/39	CAA12016
BenL	249	ketoreductase	RubG, *Streptomyces collinus*	73/56	AAG03071
BenQ	336	ketoacylsynthase III (KASIII)	FabH, *Kineococcus radiotolerans* SRS30216	65/51	EAM74678
BenX	709	integral membrane export protein	*Streptomyces avermitilis* MA-4680	72/58	BAC71120

ORF II involved in *whiE* spore pigment biosynthesis[47] and also shares high sequence identity with corresponding gene products encoded by the *rub*, *grh*, and *fdm* gene clusters, RubD, GrhS,[37] and FdmE.[42]

Tailoring Enzymes Encoded in the *ben* Gene Cluster. En route to the benastatins, the polycyclic ring system has to undergo several oxidoreduction steps to yield the reduced D ring. The deduced gene product of *benL* shows good head-to-tail similarity to enzymes of the short-chain dehydrogenase/ reductase (SDR) family (PF00106) with motifs for NADH and substrate binding. The closest orthologues of the deduced gene product of *benL* are RubG, GrhO10, and FdmO. The function of these predicted proteins has not yet been established. They might be involved in the reduction of the C19 keto group, which could lead to a preferred orientation of the polyketide chain before the fourth (D) ring is formed (Figure 6).

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The deduced gene products of *benG*, *benJ*, and the C-terminus of BenH belong to groups of putative oxidoreductases, which are also encoded in the *fdm*, *grh*, *rub*, and *pdm* gene clusters. Yet their functions are unclear. Surprisingly, inactivation of *benG* did not influence the production of benastatins, but only led to a slightly altered ratio of the individual benastatins produced (data not shown).

The last step in benastatin biosynthesis is the alkylation of the methylene bridge between rings A and C. While feeding studies revealed that two SAM-derived methyl groups are introduced at this position, only a single methyl transferase gene, *benF*, was identified in the *ben* gene cluster. The deduced gene product of *benF* contains a methyltransferase motif (pfam00891) and shows high similarity to a range of *O*-methyltransferases that utilize *S*-adenosyl methionine as methyl group donor. Functional studies revealed that BenF is capable of a rare geminal *bis*-methylation [A.S., Z.X., C.H., unpublished observa-

tions]. The cosubstrate for BenF is most likely provided by BenM, an *S*-adenosylmethionine synthetase homologue.

Molecular Basis for Starter Unit Formation and Selection. The pentyl side chain, which is composed of acetate units only, is very suggestive for the utilization of a rare hexanoate PKS starter unit. Unfortunately, all attempts to incorporate deuterium-labeled hexanoate or butyrate probes, as well as the corresponding SNAC derivatives, into benastatin by feeding experiments have failed. We thus expected to gain an insight into starter unit biosynthesis and attachment by functional analysis of the *ben* gene cluster. Surprisingly, except for *benQ*, no further genes required for fatty acid biosynthesis could be detected. The deduced gene product of *benQ* is very similar to β-ketosynthases III (KAS III, CD00830) from type II fatty acid synthase systems. The prototype KAS III, FabH from *E. coli*, is primed with the parent acyl starter unit and catalyzes the first round of Claisen condensations. KAS III enzymes feature highly conserved active site residues, Cys112, His244, and Asn274, forming a catalytic triad responsible for transacylation, decarboxylation, and condensation.[48] Homologues of these enzymes are characteristic for most aromatic polyketide pathways that are initiated with non-acetate starter units, such as anthacyclines (*akn*, *dnr*),[49] R1128 (*zhu*),[50] and frenolicin (*frn*)[51] (Figure 3). Related genes were also found in the hedamycin (*hed*)[52] and fredericamycin (*fdm*)[42] gene clusters. In addition, KSIII components (RedP, RedR) are also involved in the formation of the fatty acid portion of the prodiginines (*red*).[53] In all cases, KASIII are implicated in catalyzing the first Claisen condensation reaction for initiation of the reactions and apparently plays a crucial role in governing the total rate of the production, as well as the starter unit determination.

We thus assumed that the *benQ* gene product is the determinant for starter unit selection and attachment. To prove this, the entire *benQ* gene was deleted from cosmid p5H09 by PCR targeting[40,41] using a cassette containing a resistance marker and *oriT* (RK2), and the mutated construct was introduced into *S. albus*.[54,55] TLC and HPLC−MS analyses of the extract revealed that the metabolic profile of the resulting transconjugant, *S. albus*/pXU132, is dramatically altered (Figure 4). Interestingly, while over six new metabolites are formed, the mutant definitely lost its ability to produce any known benastatins. To prove that this result is solely due to the loss of *benQ*, the mutant was complemented. Coexpression of *benQ* on a second plasmid fully restored the native benastatin pathway (Figure 4).

Engineered Biosynthesis of Novel Benastatin Derivatives. A detailed analysis of the metabolic profile of the mutant revealed that, instead of the known benastatins, at least five novel metabolites were formed (**5**−**10**, see Figures 4 and 5). The most abundant metabolites were isolated from the mycelium of an upscaled fermentation of *S. albus*/pXU132 (14 L). Open column chromatography and repeated preparative HPLC yielded 4.4 mg of **6**, 5.6 mg of **7**, 17.2 mg of **8**, 8.7 mg of **9**, and 7.4 mg of **10**. Their structural elucidation was accomplished by EI−MS, HR−MS, IR, and extensive NMR (^{1}H, ^{13}C, HMBC, HMQC, COSY, TOCSY, NOESY) measurements. The molecular weights and the molecular compositions of **6** (502 g mol^{-1}, $C_{30}H_{30}O_7$), **7** (514 g mol^{-1}, $C_{30}H_{26}O_8$), **8** (516 g mol^{-1}, $C_{30}H_{28}O_8$), **9** (516 g mol^{-1}, $C_{29}H_{24}O_9$), and **10** (518 g mol^{-1}, $C_{29}H_{26}O_9$) were assigned from ESI−HRMS and ^{13}C NMR data. The NMR spectra of **7** and **9** strongly resemble the spectrum of benastatin A, while those of **6**, **8**, and **10** are more similar to benastatin B data.[32] The assignment of all NMR signals by DEPT135, COSY, HMQC, and HMBC experiments showed that the rings A, B, C, and D were not affected by the genetic manipulation. However, a significant change in the methylene region of the ^{1}H NMR spectra was observed. Comparison of 1D and 2D NMR data disclosed that **6** has a benastatin B-like skeleton with an unprecedented isopentyl side chain.

The investigation of the other metabolites proved to be more challenging. In addition to the benzo[*a*]naphthacene scaffold, signals of *n*-propyl moieties and the saturated or unsaturated bonding between C5 and C6 could be assigned in all products. NMR data, HR−MS, and MS^n spectra suggested that in place of the original *n*-pentyl residue a 2-oxo-pentyl chain is attached to the polyaromatic scaffold. In this case, an intramolecular ring closure by hydroxylactonization would be likely to occur. This scenario rationalizes the nondetectable NMR signals of the 16-H_2 or 16C, 17C, and the expected couplings (e.g., between 4 and 16, 16 and 18, and 17 and 18).[56] Such an "open chain-ring tautomerism" has previously been reported for more simple hydroxylactones.[57−59] Continuous interconversion between the ring and the open chain tautomeric forms hampered the interpretation of the spectra. Thus, the signals of the lactone ring were only very weak (e.g., the NMR signals C2, C3, C15−C17, or 16-H_2, see Table 2).

To affirm the proposed hydroxylactone structure, **8** was methylated using diazomethane. By means of this derivatization, the two most reactive hydroxy groups (1-OH and 11-OH) were methylated. The 7- and 9-hydroxy functions are significantly more resistant toward methylation because of the formation of strong hydrogen chelates with the 8-keto moiety.[60] Methylation was accompanied by elimination of a water molecule to form the unsaturated lactone ring, as shown by the appearance of the typical IR band of the carboxyl C═O bond at 1732 cm^{-1}.[57,61] This observation was also supported by the presence of the olefinic 16-H and C-16 that resonate at 6.17 and 102.8 ppm, respectively. The structure of **11** provided the strongest support for the proposed structures of **8** and its derivatives (**7**, **9**, and **10**). Despite various attempts, compound **5** could not be obtained in sufficient purity to allow for a full structural elucidation. However, molecular mass and ^{1}H NMR data are almost identical

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Table 2. NMR Data of Novel Benastatin Derivatives in ppm, *J* in Hz

	6		**7**		**8**		**9**		**10**		**11**	
C	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H
1	164.1		165.6		161.9		166.2		162.8		160.8	
2	112.1[a]		107.1		111.0		112.9[a]		112.9[a]		112.6	
3	149.0[a]		137.2		140.2		137.8[a]		138.1[a]		138.8	
4	121.4	6.56 (s, 1H)	120.4	7.14 (s, 1H)	121.0[a]	6.69 (s, 1H)	120.7[a]	7.22 (s, 1H)	121.5[a]	6.63 (s, 1H)	120.3	7.03 (s, 1H)
4a	144.7		140.9		148.9		140.3		140.4		149.3	
5	20.8	2.82 (m, 2H)	126.4	7.59 (d, 9.0, 1H)	20.5	2.86 (m, 2H)	126.6	7.65 (d, 9.0, 1H)	20.6	2.94 (m, 2H)	19.7	2.88 (m, 4H)
6	30.7	2.72 (m, 2H)	125.6	8.37 (d, 9.0, 1H)	30.8	2.78 (m, 2H)	125.1	8.44 (d, 9.0, 1H)	30.7	2.75 (m, 2H)	30.3	
6a	123.3		121.8		123.4		122.4		124.4		123.9	
7	159.6	12.98 (s, 1H, OH)	161.6	13.92 (s, 1H, OH)	159.7	13.02 (s, 1H, OH)	161.3	13.58 (s, 1H, OH)	159.4	12.64 (s, 1H, OH)	158.9	13.11 (s, 1H, OH)
7a	112.9		110.2		112.8		109.8		112.2		112.9	
8	191.4		191.6		191.5		191.0		190.9		190.7	
8a	108.5		108.5		108.5		108.3		108.1		108.4	
9	167.0	12.95 (s, 1H OH)	167.4	12.97 (s, 1H, OH)	167.1	12.92 (s, 1H, OH)	167.1	12.43 (s, 1H, OH)	166.7	12.48 (s, 1H, OH)	166.0	13.08 (s, 1H, OH)
10	101.9	6.21 (d, 2.2, 1H)	102.0	6.25 (d, 2.2, 1H)	101.9	6.22 (d, 2.2, 1H)	102.4	6.25 (d, 2.2, 1H)	102.3	6.21 (d, 2.2, 1H)	98.9	6.40 (d, 2.3, 1H)
11	166.2		166.9		166.4		166.9		166.6		166.5	
12	107.1	6.65 (d, 2.2, 1H)	107.5	6.72 (d, 2.2, 1H)	107.2	6.65 (d, 2.2, 1H)	106.7	7.01 (d, 2.2, 1H)	106.6	6.93 (d, 2.2, 1H)	106.3	6.67 (d, 2.3, 1H)
12a	156.2		156.2		156.2		156.7		156.5		154.5	
13	39.6		40.3		39.6		71.7	5.30 (br s, 1H, 13-O H)	71.0	5.10 (br s, 1H, OH)	39.0	
13a	149.0		148.5		149.5		148.1		149.5		149.3	
14	118.8	8.58 (s, 1H)	117.5	9.94 (s, 1H)	118.7	8.47 (s, 1H)	117.0	9.96 (s, 1H)	118.0	8.72 (s, 1H)	117.6	8.38 (s, 1H)
14a	142.6		137.4		140.5		137.5		141.5		140.2	
14b	119.9		118.8		121.3		119.3		121.3		126.8	
15	175.9[a]		173.4[a]		172.1[a]		173.7[a]		171.8[a]		163.0[a]	
16	35.2	3.12 (m, 2H)	*b*	*b*	*b*	*b*	*b*	*b*	*b*	*b*	102.8	6.17 (s, 1H)
17	42.5	1.54 (m, 2H)	*b*		*b*		*b*		*b*		159.5	
18	29.6	1.66 (m, 1H)	43.9	2.28 (t, 7.4, 2H)	44.0	2.09 (t, 7.8, 2H)	44.0	2.16 (t, 7.6, 2H)	44.1	2.21 (t, 7.8, 2H)	35.3	2.49 (t, 7.5, 2H)
19	23.1 (2C)	0.96 (d, 6.5, 6H)	17.8	1.57 (m, 2H)	17.8	1.57 (m, 2H)	17.8	1.60 (m, 2H)	17.8	1.54 (m, 2H)	20.2	1.74 (m, 2H)
20			14.5	0.89 (t, 7.4, 3H)	14.4	0.97 (t, 7.4, 3H)	14.4	0.96 (t, 7.4, 3H)	14.3	0.91 (t, 7.4, 3H)	13.5	0.99 (t, 7.4, 3H)
21	34.5 (2C)	1.69 (s, 6H)	34.9 (2C)	1.82 (s, 6H)	34.4 (2C)	1.70 (s, 6H)	39.8	1.60 (s, 3H)	39.2	1.54 (s, 3H)	34.0 (2C)	1.70 (s, 6H)
22												
1-OCH$_3$											61.6	3.71 (s, 3H)
11-OCH$_3$											55.6	3.88 (s, 3H)

[a] Weak signal. [b] Not detectable due to tautomerism.

to those of **6**, as well as the low polarity point toward the anteiso derivative (see Supporting Information).

A comparison of the new compounds with the metabolites of the wild-type producer shows that all polyketides share a pentacyclic framework. Compounds **9** and **10** are only partially methylated at position C13, whereas **7** and **8** have undergone a double alkylation catalyzed by BenF. Comparing the structures with those of the parent benastatins, it can be concluded that the minimal PKS utilized alternative starter units instead of hexanoate. Isohexanoate obviously served as the starter unit for the minor product **6**, while **7**−**10** result from priming with butyrate.

The main metabolites produced by the mutant were subjected to standardized cytotoxicity and proliferation assays. In the HeLa cell assay, the 5,6-saturated-hydroxylactone derivatives **8** and **10** show cytotoxicity (CC_{50} 9.4/11.1 μg mL^{-1}) comparable to that of benastatin A or B, and similar antiproliferative effects on L-929 mouse fibroblast cell lines (GI_{50} 8.8/11.9 μg mL^{-1}) and on human leukemia cell line K-562 (GI_{50} 10.8/10.6 μg mL^{-1}). Benastatin derivative **9** exhibited a slightly lower antiproliferative activity (L-929 GI_{50} 22.4 μg mL^{-1}; K-562 GI_{50} 24.7 μg mL^{-1}) but is significantly less cytotoxic (HeLa GI_{50} 22.8 μg mL^{-1}) as compared to benastatin A.

The formation of branched-chain benastatin analogues as well as new polyphenols with an additional ring (F) by the *benQ* null mutant allows for several conclusions regarding starter unit selectivity and chain elongation.

BenQ Is a Rigorous Determinant in Starter Unit Selection. In bacterial fatty acid biosynthesis, type III β-ketoacyl-ACP synthases (KSIII) catalyze the initial condensation of the fatty acid primer with malonyl-ACP, thereby initiating the FAS elongation cycles.[62,63] It is well known that in primary metabolism KSIII components determine the profile of fatty acids (straight/branched, even-/odd-numbered),[63,64] and pioneering work by Reynolds and co-workers revealed that altering or replacing the KSIII components opens a path for fatty acid metabolic engineering.[65,66] The occurrence of a FabH orthologue encoded in the *ben* biosynthetic gene cluster implies a similar function in the formation of the benastatins. As shown by the inactivation and complementation experiments, BenQ is responsible for providing the rare hexanoate starter unit to the *ben* minimal PKS. In the presence of this enzyme, rigorously no other starter unit is accepted. In stark contrast to the intact *ben* PKS complex, straight-chain butyryl-CoA as well as

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branched isohexanoyl-CoA are accepted by the *ben* PKS when BenQ is absent. Consequently, the FabH orthologue BenQ represents an essential determinant in starter unit selection.

The similarity of the fatty acid synthase and type II PKS systems has been the matter of numerous elaborate studies. Usually specific sets of KS, ACP, and sometimes even AT components are present in FAS, PKS priming, and PKS elongation modules. It was shown by Reynolds and co-workers that the FAS and PKS pathways function independently and that KSIII and ACP from both PKS and FAS are not interchangeable.[67] Furthermore, Tang et al. have demonstrated that even the KS in the initiation and elongation modules of aromatic polyketide synthases have orthogonal acyl carrier protein specificity.[68] In vitro studies on the substrate specificity of ZhuH by Meadows and Khosla strongly supported this model.[69] Furthermore, Khosla and co-workers succeeded in engineering modified aromatic polyketides by recombination of the R1128 loading module consisting of ZhuC, ZhuH, and ZhuG, with other min PKS components.[13,14,70] It should be noted that the additional ACP (ZhuG) is indispensable for the incorporation of non-acetate starter units in the R1128 pathway.[68] In light of these results, it is surprising that only a single ACP is encoded in the *ben* gene cluster. It is known that *Streptomyces* FabH exhibits a rather broad substrate specifity and that butyrate and isohexanoate starter units are provided to the bacterial FAS.[71−73] The absence of KSIII or ACP components from the mutated benastatin polyketide metabolic pathway could imply a cross-talk of the PKS with the FAS components. Recently, Reynolds and co-workers have made a similar observation when studying and engineering the biosynthesis of the fatty acid-derived pyrrol alkaloids prodiginines.[74] Both examples demonstrate that the interaction of KSIII systems in primary and secondary metabolite pathways may be more complex than expected. In this respect, the benastatin hybrid biosynthesis is a remarkable example for a PKS system that interacts with FAS loading enzymes.

Future work is needed to understand the specificity of the individual KSIII toward their primer units. However, the seminal work by the Stroud and Khosla groups on the crystal structure of the R1128 priming KSIII (ZhuH)[75] provided the ground for fitting the BenQ amino acid sequence and thus allowed a first glimpse into its substrate specificity. The modeled BenQ substrate binding pocket has a size similar to that of ZhuH, which is preferentially primed by isobutyryl or propionyl groups, but could also tolerate acetyl and butyryl primer units.[69,76] However, as compared to ZhuH, the cavity of BenQ is more crowded due to the voluminous side chain of His86 and the bulky aliphatic residue Met90′. These two residues represent the most obvious structural differences from the other amino acids of ZhuH stretching into the substrate binding pocket. As a result, this priming ketosynthase is unlikely to tolerate branched acyl substrates, but prefers straight-chain substrates consisting of up to four carbon atoms (see Supporting Information).

Utilization of Shorter PKS Primers Results in an Extended Ring System. Apart from the fungal norsolorinic acid synthase,[77,78] the benastatin PKS is the only known type II PKS system that utilizes a hexanoate starter. Related, albeit PKS-derived, hexadienoate primers have been reported for the *hed*[52] and *fdm*[42] pathways. The benastatins, together with fredericamycin and the griseorhodins, rank among the largest aromatic polyketides. The *ben* PKS can synthesize and accommodate a giant fatty acid−polyketide backbone formally composed of 14 acetate units. In the genuine *ben* pathway, a hexanoate starter undergoes 11 Claisen condensations. If a starter unit with shorter chain length (butyrate) is employed in the mutated system, the PKS catalyzes an additional round of elongation to yield the regular size of the polyketide backbone. This observation is in line with recent in vitro studies by Nicholson et al. using the actinorhodin synthase[79] as well as in aklanonic acid engineering experiments by Lee et al.[13,70] All of these findings provide the strongest evidence that the number of elongation steps is controlled by the total size of the polyketide chain that can be accommodated in the cavity of the enzyme complex. Fascinating structural work by the Stroud and Khosla work groups lend strongest support for this model, and even identified potential gatekeepers of the tunnel.[9] Elegant previous starter unit engineering experiments have resulted in novel metabolites, albeit in all cases the products were derived from longer starter units and thus feature reduced ring systems.[13,14] The example reported in the present study is novel because a shorter starter has been utilized, and the keto group from incorporation of the non-natural butyrate starter gives rise to an additional ring (F). Interestingly, the enlarged ring system of benastatin derivative **11** is similar to the known natural compound fredericamycin C_1 produced by *Streptomyces griseus*[80] and collinone, which has been isolated from a strain expressing an incomplete set of rubromycin biosynthesis genes.[43] Both fredericamycin C_1 and collinone have been implied as intermediates (or shunt products) in pathways leading to the structurally intriguing spiro compounds. The dehydrated benastatin derivative neatly fills the gap between the trideca- and pentadecaketides (Figure 7) and corroborates the proposed biosynthetic pathways. From the engineering point of view, our work demonstrates that by a facile manipulation and elongation of ketide chains the polyketide ring systems can be extended.

In conclusion, the molecular basis for the biosynthesis of the benastatins, potent antitumor agents and inducers of apoptosis, has been elucidated. The entire benastatin biosynthesis gene cluster was cloned, sequenced, and its identity was confirmed by heterologous expression. The boundaries of the cluster were determined by deletion of flanking regions. As shown by inactivation and complementation, BenQ, a KSIII, is crucial for the incorporation of the rare hexanoate starter unit. From a

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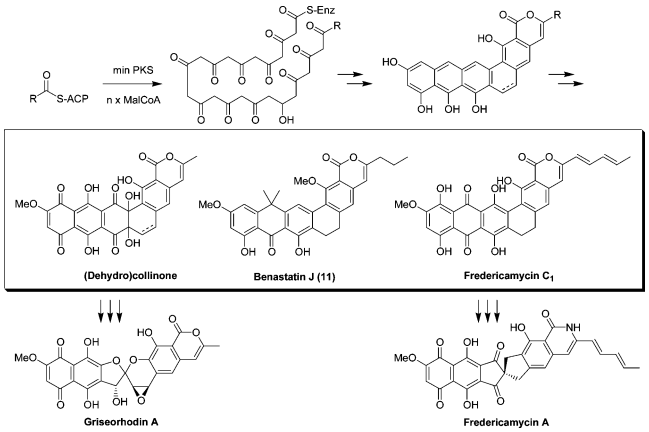

Figure 7. Comparison of benastatin derivative **11** with proposed intermediates from the griseorhodin and fredericamycin pathways.

mutant lacking *benQ*, five novel antiproliferative benastatin derivatives were isolated and fully characterized. It is remarkable that the *ben* PKS is capable of accepting typical straight-chain and branched-chain fatty acid synthase primers. The length of the polyketide backbone is increased when shorter straight-chain starters are utilized, and an extended, hexacyclic ring system is formed that is reminiscent of proposed griseorhodin and fredericamycin pathway intermediates. These experiments illustrate the potential of polyketide pathway engineering to yield new polyphenols with altered substituents and/or ring topologies. Furthermore, the *ben* biosynthetic pathway now serves as an ideal model for the systematic functional analysis of griseorhodin and fredericamycin pathways enzymes, and to employ these tools for rationally designing novel aromatic polyketides.

Experimental Section

General. Mass spectra were obtained from a LCQ electrospray MS (Thermo Electron). High-resolution MS spectra were recorded on a MAT 95 XL (Thermo Electron). One and two-dimensional ^{1}H and ^{13}C NMR spectra were recorded on a Bruker ADVANCE dpx 300 and drx 500, respectively, in THF-d_8 (if not indicated otherwise) using the solvent signals as reference. IR spectra were obtained on a Satellite FTIR manufactured by Mattson. Thin layer chromatography was performed on silica gel 60 F_{254} plates (layer thickness 0.2 mm, Merck). For column chromatography, silica gel 60, 40−63 μm, treated with aqueous 0.1 M KH_2PO_4, was used. Preparative HPLC separation was performed on a Machery-Nagel Nucleosil 100-7, C-18 (20 × 250 mm) column, and the analytic HPLC was performed on a Chrom Nucleosil 100-5, C-18 (4.6 × 250 mm) column. Flow rate: 15 mL min^{-1} (preparative HPLC), 1 mL min^{-1} (analytical HPLC).

Bacterial Strains and Culture Conditions. Benastatin producer *Streptomyces* sp. A2991200 was kindly provided by Dr. P. Krastel (Novartis) and was used as the source of DNA in the construction of the genomic DNA library. *S. lividans* TK23 and *S. albus* (kindly provided by Prof. J. Salas) served as host strains for heterologous expression experiments. For benastatin production, wild-type and mutant strains were cultivated in MS medium (mannitol soya flour medium)[5] for 6 days at 30 °C with shaking. *S. lividans* TK23 was cultivated on R5 agar and YEME liquid medium[5] for protoplast transformation and on MS for all other experiments. Transformants were selected with thiostrepton at 5 μg mL^{-1}, and/or apramycin at 50 μg mL^{-1}, and/or spectinomycin at 400 μg mL^{-1} in both solid and liquid medium and depended on different requirements. *E. coli* strains EPI100-T1 (Epicentre) and XL1 blue served as hosts for library construction and routine subcloning, respectively. For intergenic conjugation, *E. coli* ET12567 containing the RP4 derivative pUZ8002 was used.[5] *E. coli* strains were grown in LB medium supplemented with ampicillin (100 μg mL^{-1}), or apramycin (50 μg mL^{-1}), or spectinomycin (50 μg mL^{-1}) for selection.

Plasmids and General DNA Procedures. DNA isolation, plasmid preparation, restriction digests, gel electrophoresis, and ligation reactions were conducted according to standard methods.[5,81] pBluescript II SK(-), pGEM T-easy (Promega), and p*MOS*Blue (Amersham) were the routine vectors for subcloning and preparation of DNA templates for sequencing. The *E. coli*−*Streptomyces* shuttle vector pKJ01[15] was used for genomic DNA library construction and heterologous expression in *Streptomyces*. Restriction enzyme-digested DNA fragments were recovered from agarose gel by the GFX PCR DNA and Gel Band Purification Kit (Amersham). For Southern blot hybridization, the DIG (Roche) and ECL (Amersham) DNA Labeling and Detection Kits were used. Sequencing of p5H09 was performed in a shotgun approach. Remained gaps were filled by targeted subcloning and primer walking.

Construction and Screening of a *Streptomyces* sp. A2991200 Genomic Cosmid Library. A *S.* sp. A2991200 genomic DNA library was constructed in *E. coli* EPI100-T1 (Epicentre) using pKJ01 as cosmid vector.[15] For DNA extraction, the "rapid small scale isolation of *Streptomyces* total DNA" method[5] was applied, and then the DNA was randomly sheared by expelling from a syringe through a small bore needle and isolated from low melting point agarose gel, yielding fragments with an average size around 40 kb. Components of pWEB Cosmid Cloning Kit (Epicentre) were used to finish the construction of the genomic library. Hybond N^+ (Amersham Pharmacia) was utilized for the screening of 1920 cosmid clones and further identification of the positive cosmid with a *grh* KSα gene probe[37] following standard hybridization procedures. The sequence of the *ben* biosynthesis gene cluster has been deposited in the EMBL database under accession number AM501485.

Heterologous Expression of the *ben* Biosynthetic Gene Cluster. Positive cosmids obtained from screening of the genomic cosmid library by Southern hybridization were introduced intro *S. lividans* TK23 for heterologous expression. p5H09 was the only cosmid that conferred to the host the ability of producing benastatins. The production was identified by HPLC and MS as compared to that of the wild-type strain. Cosmid pXU130 that contains essential genes for the biosynthesis of benastatins was obtained from deletion of the flanking region downstream of *ben*K on p5H09 via the λRed system.[40,41] Introduction of pXU130 into *S. albus* by conjugation yielded a benastatin-producing strain.

Inactivation of *benQ*. The *benQ* deletion mutant was constructed by PCR targeting based on the λRed method.[40,41] To amplify the extended streptomycin and spectinomycin resistance gene (*aadA*) flanked by FRT sites (FLP recognition targets) and 39 nt from sense/antisense strands ending in start/stop codons, two long primers for each gene were designed: benQ_PTL, 5′-CATCACCTCGCAGGTGATCCGTATGAAGGAGCGCAGATGATTCCGGGGATCCGTCGACC-3′ (59nt); and benQ_PTR, 5′-ACAGCACGACCGAAGGCCGTCGCTGACAGGCGGTCCCTATGTAGGCTGGAGCTGCTTC-3′ (58nt) for *ben*Q. The amplicons were introduced into *E. coli* BW25113/pIJ790 containing cosmid p5H09 with concomitant substitution of *benQ* by the extended antibiotic resistance cassette. The inserted cassette was maintained in the cosmid for the sake of facilitating conjugation. The resulting construct, pXU132, was introduced into *S. albus* by intergenic conjugation using *E. coli* ET12567 containing the RP4 derivative pUZ8002. The transconjugants were selected for their thiostreptone resistance.

Cloning and Heterologous Expression of *benQ* for Complementation. *benQ* was amplified from cosmid p5H09 by PCR, followed by sequencing and subcloning steps. Two modified primers with recognition sites for restriction enzymes *Bam*HI (GGATCC) and *Eco*RI (GAATTC), benQ_CPL, 5′-CTCGCAGGGGATCCGTATGAAGG-3′,

(81) Sambrook, J.; Fritsch, E. F.; Maniatis, T. *Molecular Cloning: a Laboratory Manual*, 2nd ed.; Cold Spring Harbor: New York, 1989.

and benQ_CPR, 5′-CGTCCGATGAATTCGGACCTTGC-3′, were designed for amplication of *benQ*. The 1.1 kb fragment was ligated into the *Bam*HI and *Eco*RI sites of pXU56, downstream of oriT for conjugation and a constitutive resistance gene promoter from pIJ778. The resulting cassette was further cloned into the *Xba*I and *Eco*RI sites of an *E. coli*−*Streptomyces* shuttle vector pEM4A (kindly provided by Prof. Salas), yielding pXU110.

Fermentation of *S. albus*/pXU132 and Isolation of Novel Benastatin Derivatives (5−10). Spores of strain *S. albus*/pXU132 were used to inoculate 100 mL of a seed medium (15 g L^{-1} glucose, 15 g L^{-1} soy bean meal, 5 g L^{-1} NaCl, 1 g L^{-1} $CaCO_3$, 0.3 g L^{-1} KH_2PO_4) in 250 mL Erlenmeyer flasks, supplemented with thiostreptone (5 mg L^{-1}) for antibiotic selection, and cultivated at 28 °C for 2 d on a rotary shaker (180 rpm). 12 mL aliquots of the seed culture were transferred into 250 mL portions of 14 L of production medium (20 g L^{-1} mannitol, 20 g L^{-1} soy bean meal) in 1 L Erlenmeyer flasks and cultivated at 28 °C for 4 d on a rotary shaker (180 rpm).

To isolate the secondary metabolites, the culture broth was separated into the mycelial cake and the culture filtrate. The mycelial cake was extracted with methanol (4 L). The extract was concentrated under reduced pressure to an aqueous solution. It was re-extracted with ethyl acetate, and the organic phase was dried over anhydrous Na_2SO_4 and concentrated under reduced pressure, yielding 135 mg of mycelial crude extract. The culture filtrate was exhaustively extracted with ethyl acetate, and the organic phase was dried and concentrated under reduced pressure, yielding 2.3 g of extract.

The combined extracts (brown oil) were subjected to open column chromatography on silica gel using a chloroform/methanol (100:0−50:50) gradient for elution. Benastatin-containing fractions were subjected to further purification by preparative RP-HPLC using 0.1% AcOH in acetonitrile/0.1% AcOH in water (60:40−100:0). Preparative HPLC afforded **6** (4.4 mg), **7** (3.8 mg), **8** (17.2 mg), **9** (8.7 mg), and **10** (7.4 mg) as yellow substances. The purity of the obtained fractions was tested by analytical HPLC using 0.1% AcOH in acetonitrile/0.1% AcOH in water (60:40−100:0) gradient.

6 (Benastatin E). For NMR data, see Table 2. IR (powder): ν 3648, 2952, 2925, 2855, 1670, 1597, 1464, 1365, 1283, 1152, 1029, 827 cm^{-1}. ESI−MS (*m*/*z*, %): 501 ([M − H]$^-$, 100), 457 ([501 − CO_2]$^-$, 20), 442 ([M − 60]$^-$, 5). HRMS calcd for $C_{30}H_{29}O_7$, 501.1908; observed, 501.1903.

7 (Benastatin F). For NMR data, see Table 2. IR (powder): ν 3234, 3066, 2953, 2924, 2854, 2699, 1617, 1596, 1464, 1442, 1362, 1278, 1152, 1030, 833, 81 cm^{-1}. ESI−MS (*m*/*z*, %): 513 ([M − H]$^-$, 100), 469 ([515 − CO_2]$^-$, 15), 454 ([M − 60]$^-$, 10). HRMS calcd for $C_{30}H_{25}O_8$, 513.1544; observed, 513.1485.

8 (Benastatin G). For NMR data, see Table 2. IR (powder): ν 3628, 3222, 2957, 2924, 2854, 1716, 1599, 1462, 1429, 1364, 1274, 1179, 1029, 765 cm^{-1}. ESI−MS (*m*/*z*, %): 515 ([M − H]$^-$, 100), 471 ([515 − CO_2]$^-$, 25), 456 ([M − 60]$^-$, 8). HRMS calcd for $C_{30}H_{27}O_8$, 515.1700; observed, 515.1687.

9 (Benastatin H). For NMR data, see Table 2. IR (powder): ν 3649, 3335, 3081, 2960, 2926, 2872, 1618, 1602, 1457, 1376, 1266, 1177, 1049, 820 cm^{-1}. ESI−MS (*m*/*z*, %): 515 ([M − H]$^-$, 100), 471 ([515 − CO_2]$^-$, 8), 456 ([M − 60]$^-$, 18). HRMS calcd for $C_{29}H_{23}O_9$, 515.1337; observed, 515.1311.

10 (Benastatin I). For NMR data, see Table 2. IR (powder): ν 3649, 3323, 3065, 2958, 2872, 1604, 1457, 1431, 1374, 1266, 1160, 1050, 994, 775 cm^{-1}. ESI−MS (*m*/*z*, %): 517 ([M − H]$^-$, 100), 473 ([517 − CO_2]$^-$, 5), 458 ([M − 60]$^-$, 15). HRMS calcd for $C_{29}H_{25}O_9$, 517.1493; observed, 517.1466.

Methylation of 8. Compound **8** (0.025 mmol, 12.7 mg) was dissolved in 3 mL of methanol. 3 mL of etheral diazomethane, generated from 10.3 g of *N*-methyl-*N*-nitroso-urea in 100 mL of ether, was added to the solution, and the mixture was left standing overnight. The mixture was concentrated in vacuo and repeatedly separated by preparative RP-HPLC, using acetonitrile/0.1% TFA in water (60:40−100:0), affording 2.8 mg of **11** (Benastatin J), among minor side products. For NMR data of **11**, see Table 2. IR (powder): ν 2963, 2929, 2871, 2850, 1732, 1658, 1597, 1468, 1423, 1401, 1372, 1307, 1277, 1252, 1214, 1162, 1141, 1042, 998, 754 cm^{-1}. ESI−MS (*m*/*z*, %): 525 ([M − H]$^-$, 100), 495 ([525 − 2 × CH_3]$^-$, 3). HRMS calcd for $C_{32}H_{29}O_7$, 525.1950; observed, 525.1913.

Biological Assays. Cytotoxic and antiproliferative assays using HeLa, L-929 mouse fibroblast, and K-562 human leukaemia cell lines were conducted as described in detail in refs 82 and 83.

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Supporting Information Available: Superposed binding pockets of BenQ and ZhuH and tentative structure of **5**. This material is available free of charge via the Internet at http://pubs.acs.org.

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