ON THE BIOSYNTHESIS OF ASPERLICIN AND THE DIRECTED BIOSYNTHESIS OF ANALOGS IN *ASPERGILLUS ALLIACEUS*

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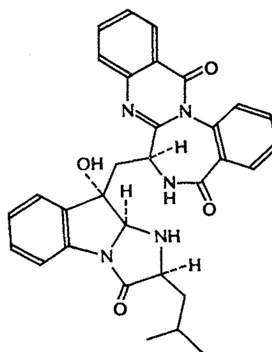
Feeding of ^{14}C-labeled amino acids to resting cells of *Aspergillus alliaceus* strongly supported the intuitive hypothesis that asperlicin is biosynthesized from tryptophan, anthranilate and leucine. The resting cell system was used also to prepare 25 asperlicin analogs *via* directed biosynthesis in presence of analogs of tryptophan and leucine.

Asperlicin (1), a metabolite of *Aspergillus alliaceus*, is a non-peptide antagonist of the cholecystokinin receptor[1]. The fermentation, isolation, and biological properties of this natural product were described in a series of reports by researchers at Merck[1~3]. This natural benzodiazepine appears to be composed of tryptophan, leucine and two anthranilate moieties. Two minor metabolites, asperlicin E (2) and asperlicin C (3), produced in the same fermentation[4] lack the leucine moiety. All three compounds are related to other fungal metabolites such as tryptoquivaline[5] and auranthine[6].

This paper describes our studies on the biosynthesis of asperlicin and the minor components, 2 and 3. In addition, we have used the biosynthetic information to construct derivatives of the fungal products; analogs of 1, 2, and 3 were produced by resting cells in the presence of analogs of tryptophan and leucine. The production, isolation, and identification of these biosynthetic analogs are described in this report.

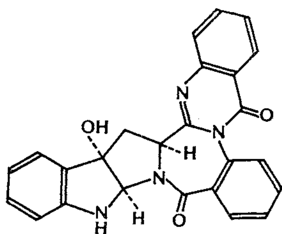


Asperlicin

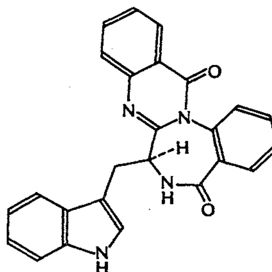
Experimental

Materials

The common amino acids and the following compounds were purchased from Sigma: DL- and



Asperlicin E



Asperlicin C

L-allylglycine, L-ethionine, L-norleucine, α-L-phenylglycine, DL-6-methyltryptophan, and DL-5-methyltryptophan. DL-5-Fluorotryptophan and DL-6-fluorotryptophan were obtained from Aldrich. United States Biochemicals supplied DL-7-azatryptophan. DL-4-Methylallylglycine and DL-cyclohexylglycine were purchased from ICN. DL-Trifluoromethylleucine and DL-2-[3-cyclopentenyl]glycine were obtained from the in-house chemical collection at Merck & Co. Radiolabeled compounds were obtained from the following sources: L-[*U*-^{14}C]Leucine (347 mCi/mmol) from NEN, L-[*methylene*-^{14}C]-tryptophan (45 mCi/mmol) and L-[*G*-^{3}H]tryptophan (8.5 Ci/mmol) from Amersham, [*carboxy*-^{14}C]-anthranilic acid (46 mCi/mmol) from RPI, and DL-[7a-^{14}C]tryptophan (3.5 mCi/mmol) from ICN.

Fermentation and Resting Cells

The strain of *A. alliaceus* used in this study (ATCC 20738) is a natural reisolate of ATCC 20655. A lyophilized tube of *A. alliaceus* was used to inoculate 18×175 mm agar slants containing 15 ml of medium A. After 3 weeks of growth at 27°C, the spores from a slant tube were harvested by scraping into 6 ml 0.1% sodium lauryl sulfate. From the resulting spore suspension, 2 ml was used to inoculate 40 ml of medium B in a 250-ml three-baffle flask. The seed culture was incubated at 27°C on a 220-rpm rotary shaker for 28 hours and then used to provide a 2.5%-inoculum for 40 ml mediums C and D in 250-ml unbaffled flasks. Cultures in medium D were incubated on a 220-rpm rotary shaker at 27°C for 6 days after which time the broth was assayed for **1**. For the preparation of resting cells, cultures in medium C were grown for 48 hours and then washed three times with two volumes of 50 mM MES (3-(*N*-morpholino)ethanesulfonic acid, sodium salt), pH 6.5. Cells were always collected by centrifugation (3,000 rpm) in this procedure; the final working suspension consisted of approximately 24 mg of cells in 1 ml buffer (2×original cell concentration).

The initial screen for novel biosynthetic products involved small scale incubations in 25×150 mm tubes; 2 ml of the cell suspension was combined with 2 ml of the buffer containing common amino acids (Phe, Trp, Leu) and/or amino acid analogs. After 24 hours on a 220-rpm rotary shaker at 27°C, the 4-ml broths were extracted with two 5-ml portions of EtOAc. The solvent was evaporated under a stream of nitrogen and the residue was dissolved in MeOH. HPLC analysis was used to detect **1**, **2**, **3** and potential analogs.

Production of analogs for isolation was accomplished with larger volumes of washed cells: A seed culture, in medium B, was used to inoculate 400 ml of medium C in a 2-liter, unbaffled flask. The culture was grown for 48 hours and the mycelia were washed as described above. In sterile 250-ml flasks (unbaffled), 20 ml of cell suspension was combined with 20 ml of buffer (pH 6.5) which contained selected natural amino acids and/or amino acid analogs. The exact conditions of the fermentations are given in Table 3. After 36 hours of incubation, the whole broth was extracted twice with 1.5 volumes EtOAc; the analogs of **1**, **2**, and **3** were isolated from the organic extract.

The following media were used in this study: Medium A was composed of (g/liter) yeast extract (4), malt extract (4), glucose (10), and agar (20). This slant medium was adjusted to pH 7.0 before sterilization. Medium B was (g/liter) glutamate (5), lecithin (1), glycerol (15), NH_4Cl (2), K_2HPO_4 (2), KCl (0.5), $MgSO_4 \cdot 7H_2O$ (0.5), $ZnSO_4 \cdot 7H_2O$ (0.01), and $FeSO_4 \cdot 7H_2O$ (0.01) in distilled water at pH 6.5. Production medium C was identical to B except algin (1 g/liter) was substituted for lecithin. Production medium D was medium C plus L-tryptophan (2.0 g/liter) and phenylalanine (2.0 g/liter).

Radiolabeling Studies

Radioactive amino acids were incubated with resting cells (4 ml) for 20 hours. The conditions are given in Table 2. The EtOAc extract (2×4 ml) was evaporated under a stream of nitrogen and the resulting residue was dissolved in 0.5 ml MeOH. The specific radioactivity of a natural product was determined by HPLC: Integration of the peaks at 254 nm vs. ^{14}C dpm (Flow I model-H5, radioactivity detector).

Compounds **1**, **2**, and **3** were labeled with tritium by overnight (12 hours) incubation of resting cells (5 ml) with L-leucine (3 mg/ml), L-phenylalanine (3 mg/ml) and 300 μCi L-[*G*-^{3}H]tryptophan. After extraction into EtOAc, the products were isolated by semipreparative HPLC; Altex ODS (5 μm, 1×25 cm), 38% CH_3CN in H_2O at 3 ml/minute. The specific activities of [^{3}H]-**1**, [^{3}H]-**2**, and [^{3}H]-**3** were on the order of 0.5 μCi/μmol. Each radiolabeled compound was fed back to resting cells; ap-

proximately 20,000 dpm (in 10 µl MeOH) was added to 4 ml cells along with leucine, phenylalanine, and tryptophan (all 20 mM). Two incubations (in tubes) were extracted with EtOAc at 1 hour intervals for 8 hours and then at 20, 21, 22, 23, and 24 hours. The distribution of ^{3}H was determined by HPLC.

HPLC

HPLC was used as a quantitative method for determining the titers of **1**, **2**, and **3** in fermentation broths. In addition, HPLC was used to detect and purify biosynthetic products. Two analytical systems were used in this study. System 1 was used for routine analysis of **1~3** and to detect novel compounds derived from the leucine analogs (compounds **17** to **28**): Beckman model 342 system equipped with model 165 UV detector (235 and 210 nm), Altex 5 µm-C_8 column (4.6×250 mm), isocratic elution with 40% aqueous CH_3CN at 1 ml/minute. System 2 was used to detect compounds derived from the tryptophan analogs (**4~16**): Zorbax C_{18} column (4.6×250 mm), isocratic elution with 40% aqueous CH_3CN at 2 ml/minute (40°C), and detection at 254 and 224 nm with a Micromeretics dual wavelength detector. The capacity factors (k′) for the analogs and the natural products are shown diagramatically in Fig. 2. Conditions for preparative isolation of analogs **17** through **28** were: Altex 5 µm-C_8 (1×25 cm), 40% aqueous CH_3CN at 3 ml/minute with detection at 254 nm. Conditions for preparative isolation of **4~16** were: Zorbax C_{18} (22×250 mm), 40% aqueous CH_3CN at 10 ml/minute.

MS and NMR Conditions

MS were recorded on a Finnigan-Mat 212 instrument at 90 eV in the electron impact (EI) mode. Exact mass measurements were made on the same instrument using perfluorokerosene as the internal standard and the peak matching method. Trimethylsilyl derivatives were prepared with a 1:1 mixture of bistrimethylsilyltrifluoroacetamide and pyridine at 50°C for 1 hour. Selected samples were characterized by ^{1}H NMR in $CDCl_3$ at 300 MHz on a Varian SC-300 spectrometer.

Results and Discussion

Fermentation and Resting Cells

The original media designed for the asperlicin fermentation[2)] were not useful for our biosynthesis work, mainly because they contained complex ingredients and failed to support titers greater than 50 mg/liter. Thus, we developed a defined medium that routinely yielded asperlicin at levels of 100 to 400 mg/liter after 144 hours of fermentation. The final titer depended heavily upon the nature of the carbon source and presence (or absence) of three aromatic amino acids; phenylalanine tryptophan, and anthranilate. In all cases, production of **1**, **2**, and **3** initiated at 24 hours (post-inoculation) and promptly terminated at 144 hours. One early conclusion was that glucose repressed the biosynthesis of these natural products; addition of 1 to 3% glucose to medium D decreased titers 6- to 30-fold. Increasing the glycerol content above 12 g/liter also inhibited production. Medium D, which included Phe and Trp, supported a

Table 1. Influence of amino acids on the production of asperlicin (**1**) and related metabolites (**2** and **3**) by resting cells of *Aspergillus alliaceus*.

Amino acids (10 mM)	Titers (µg/ml) at 24 hours		
	1	**2**	**3**
None	Trace	ND	ND
Phe	26	2	ND
Trp	17	2	ND
Anthranilate	12	2	ND
Tyr	8	ND	ND
Leu	8	ND	ND
Glu	8	ND	ND
Phe+Leu	26	2	ND
Trp+Leu	40	3	ND
Phe+Trp	94	12	Trace
Phe+Trp+Leu[a]	115	10	4
Trp+ethionine[b]	16	10	16

[a] The titer of **1** after 48 hours was 210 µg/ml.
[b] Ethionine at 2 mg/ml; also was converted to compound **23** (2 µg/ml).
ND: Not detected.

titer of 400 mg/liter. Without the aromatic amino acids (medium C), titers ranged from 10 to 20 mg/liter. Addition of leucine to the media had little or no effect on production. Anthranilic acid increased production in both medium C (10%) and medium D (30%). The titers of **2** and **3** were usually 6 and 3 mg/liter, respectively.

Additional information on the control of asperlicin production was obtained from experiments with resting cells. In our standard protocol, washed cells were prepared from cultures that were grown for 40 hours in medium C (no aromatic amino acids). Without an exogenous source of amino acids, resting cells produced only a trace of asperlicin and no detectable levels of **2** and **3** after 24 hours of incubation (Table 1). Considering the structures of the natural products, it was not surprising to find that the aromatic amino acids significantly influenced the overall production. Although tyrosine had no effect, phenylalanine had a greater positive influence than the two structural components of **1**, tryptophan and anthranilate. Since the resting cells were, by definition, not growing, we expected that the aromatic amino acids were influencing production simply at the substrate level. Indeed, resting cells that had been grown in the presence of Phe, Trp and anthranilate, did not have an increased capacity (rate or total material) for asperlicin production when compared to cells grown exclusively on glycerol and glutamate. This suggests that the aromatic amino acids do not influence expression of the biosynthetic genes. Phenylalanine could have a direct effect on the biosynthetic enzymes or the enzymes of the Phe and Trp pathways; for example, it might inhibit chorismate mutase and/or activate anthranilate synthase (which would provide more precursor for biosynthesis). In any case, the limiting factor for the biosynthesis of **1** by resting cells appears to be the availability of aromatic amino acid precursors.

Biosynthesis

First of all, we wanted to obtain some experimental evidence for the biochemical origins of asperlicin and the related fungal metabolites. This was accomplished by feeding ^{14}C-labeled amino acids to resting cells and determining the specific radioactivity of the resulting product, **1**. The results summarized in Table 2, supported the intuitive hypothesis that asperlicin is constructed from amino acid building blocks: Tryptophan, anthranilate, and leucine. Both leucine and anthranilate were incorporated into **1** with little or no dilution of ^{14}C-label. Note that since anthranilate is both a precursor and a metabolite of tryptophan, each molecule of asperlicin could be labeled by three molecules of ring-labeled tryptophan (theoretical maximum incorporation 3.0) or two molecules of [*carboxy*-^{14}C]-anthranilate (theoretical maximum incorporation 2.0). [*Methylene*-^{14}C]tryptophan can directly label asperlicin only on a one-to-one basis. A specific incorporation of greater than 1.0 indicated that under nutrient-limiting conditions, some of the tryptophan was catabolized (to anthranilate) and that the

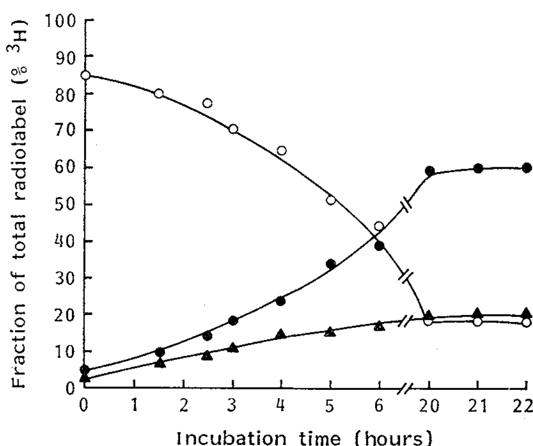
Table 2. Incorporation of ^{14}C-labeled amino acids into asperlicin.

Amino acid (10 mM)	μCi/μmol	Asperlicin[a]		
		μmol	μCi/μmol	Molar specific incorporation
DL-[7a-^{14}C]Tryptophan	0.015	0.09	0.033	1.5
L-[*Methylene*-^{14}C]tryptophan	0.018	0.10	0.025	1.4
[*Carboxy*-^{14}C]anthranilate	0.064	0.06	0.058	0.9
L-[*U*-^{14}C]Leucine	0.021	0.03	0.019	0.9

[a] Asperlicin was isolated from 4 ml resting cells after 20 hours.

side chain carbons were made available for biosynthesis. In fact, addition of 10 mM leucine to the incubation with [*methylene*-^{14}C]tryptophan, decreased the specific incorporation to 0.7. Leucine did not dilute the incorporated radioactivity from ring-labeled tryptophan. The labeling data encouraged us to use the resting cell for directed biosynthesis with analogs of tryptophan, leucine, and anthranilate. In fact, it was the analog study, described in the next section, that unequivocally defined the precursor-product relationships. Recently, ^{14}C-labeling experiments by YEULET and MANTLE provided evidence that auranthine is constructed from glutamine and anthranilate molecules[6].

Fig. 1. Bioconversion of tritium-labeled asperlicin C (**3**) (○) to asperlicin (**1**) (●) and asperlicin E (**2**) (▲) by resting cells of *Aspergillus alliaceus*.



The incubations consisted of cells (4 ml) plus Leu, Phe, Trp (all 10 mM) and [^{3}H]-**3** (7 µg, 20,000 dpm). The ethyl acetate extracts (two tubes per point) were concentrated (into 0.2 ml MeOH) and analyzed by HPLC (20 µl injections). At t=0 minute, only **3** could be detected by UV; the added label was approximately 85% **3**, 7% **1**, and 7% **2**. After 24 hours the titers were **1** 150 µg/ml, **2** 13 µg/ml, and **3** 4 µg/ml.

Next, we wanted to study biosynthetic relationships of the three natural products; the structural features of asperlicin C suggested that it may be a direct precursor to both asperlicin and asperlicin E. This hypothesis was tested by following the metabolism of ^{3}H-labeled **1**, **2**, and **3** in resting cells. The compounds were tagged with tritium by incubating L-[*G*-^{3}H]tryptophan with cells, and then isolated by the usual procedure of organic extraction followed by HPLC. Each tritiated metabolite was then fed back to resting cells that were actively producing asperlicin from Phe, Trp, and Leu. The fate of tritium was determined by HPLC; a radioactivity detector was placed in series with the UV detector. Fig. 1 clearly shows that **3** was converted to both **1** and **2** over a 24 hours period. In parallel experiments, resting cells did not metabolize either [^{3}H]-**1** or [^{3}H]-**2**. Thus **1** and **2** appear to be end products. It is important to note that at each data point, more than 85% of the total radiolabel was recovered within the pool of the three metabolites (the efficiency of the extraction was approximately 90%). Therefore, asperlicin is produced by a condensation of **3** with leucine, followed by hydroxylation (from O_2?) of indole carbon-3. An intramolecular condensation of **3** generates **2**. Obviously the intermolecular reaction with leucine is the predominant pathway. Considering that the cells are able to excrete and assimilate biosynthetic intermediates, the asperlicin system promises to be ideal for studying mutants that are blocked in the biosynthesis of a fungal product.

Directed Biosynthesis of Analogs

The ^{14}C-labeling studies demonstrated that the resting cells were very efficient at incorporating natural precursors into asperlicin. Thus, we attempted to use the existing biosynthetic machinery for the incorporation of precursor analogs into the basic structures of **1**, **2**, and **3**. The screen was simple; analogs of tryptophan, phenylalanine, leucine, or anthranilate were incubated (usually 1 mg/ml) with a small volume of resting cells (4 ml). The incubations were carried out with and without appropriate cosubstrates. For instance, leucine analogs were incubated with Phe and/or Trp. The exact in-

Table 3. Conditions for the directed biosynthesis of analogs **4~28** by resting cells of *Aspergillus alliaceus*.

Amino acid substrate (mg/ml)	Cosubstrate (mM)	Total volume (ml)[a]	Analogs produced
DL-6-Fluorotryptophan (1.0)	None	200	**10~12**
DL-5-Fluorotryptophan (1.0)	None	200	**4~9**
DL-7-Azatryptophan (1.0)	None	200	**13**
DL-6-Methyltryptophan (1.0)	L-Leucine (20)	360	**14**
DL-5-Methyltryptophan (1.0)	L-Leucine (20)	360	**15** and **16**
DL-5-Trifluoroleucine (4.0)	L-Phenylalanine (20)	360	**17** and **18**
DL-3-Methylallylglycine (4.0)	L-Phenylalanine (20)	360	**19**
DL-Allylglycine (4.0)	L-Phenylalanine (20)	240	**20** and **21**
L-Isoleucine (4.0)	L-Phenylalanine (20)	400	**26**
L-Norleucine (4.0)	L-Phenylalanine (20)	400	**27**
L-Methionine (4.0)	L-Tryptophan (20)	400	**28**
DL-2-[3-Cyclopentenyl]glycine (2.0)	L-Tryptophan (20)	120	**22**
L-Ethionine (4.0)	L-Tryptophan (20)	400	**23**
α-DL-Cyclohexylglycine (1.0)	L-Tryptophan (40)	240	**24**
α-L-Phenylglycine (4.0)	L-Tryptophan (20) and L-phenylalanine (6)	500	**25**

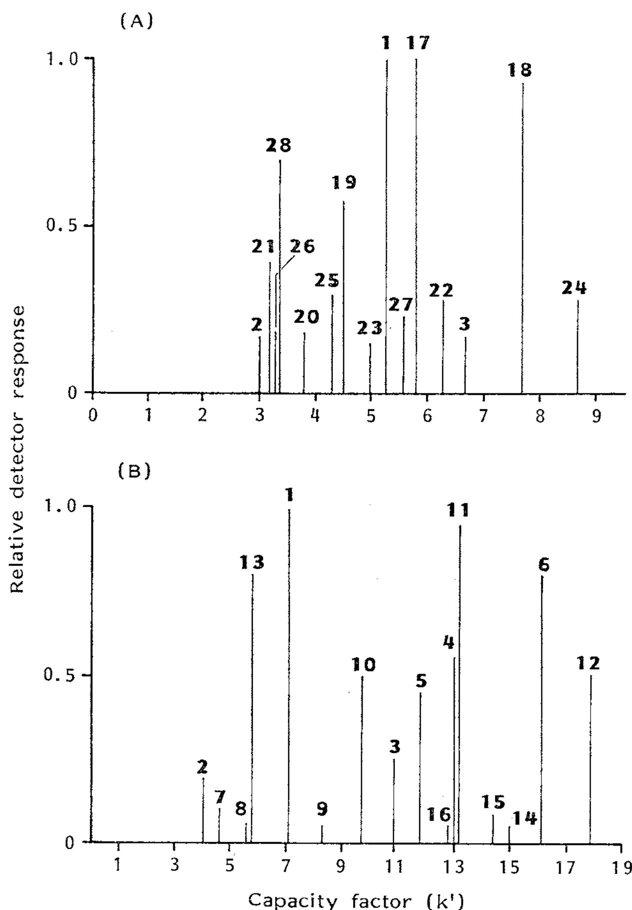
[a] Fermentation volumes are multiples of the 40 ml production cultures.

cubation conditions for the analogs that produced new compounds are given in Table 3. After 24 and 48 hours, the products were partitioned into ethyl acetate; HPLC was used to detect novel components related to asperlicin (Fig. 2). "New peaks" were pursued only when the titer was estimated to be greater than 0.1 mg/ml. For the isolation of novel compounds, the incubations were repeated on larger scales (200 to 1,000 ml) and extracted with ethyl acetate; HPLC conditions were modified for maximum resolution of the novel components on preparative columns.

The structural assignments were made by comparison with previously published MS and NMR data[3,4]. The molecular formulae were determined by high resolution MS and the exact mass values were in accord with calculated values. Tables 4~6 indicate the structure, empirical formula and characteristic MS fragment ions used to identify the point of modification. A brief description of the fragment ions used, the assumptions made, and any ambiguities which were found are given below.

For simplicity the three aromatic regions within the compounds are referred to as A1, A2, and A3, where A1 is the indole moiety, A2 is the anthranilate in the six-six ring system, and A3 is the anthranilate in the six-seven ring system. Fragment 1 defines the condensed bisanthranilate containing both A2 and A3. Fragment 2 includes indole (A1) plus the leucine derived residue. As indicated in the previous paper[4], the fragmentation scheme is slightly changed for the asperlicin E compounds. Note that although the A2 and A3 region is defined by fragment 1 in the asperlicin E compounds (Table 5), the mechanism for this loss is described differently than for the asperlicin and asperlicin C derived compounds (Tables 4 and 6). Fragment 3, which is distinct in the asperlicin E derived samples, results from cleavage at the A2 region and thus defines the A1 and A3 regions. This was important in assigning the structure of compound **7** in that it allowed for the placement of the fluorine in A3. The placement of the fluorine in A2 or A3 was not as unambiguous in structures **4** and **6** (Table 4). Here it was necessary to rely on the intensity of the 148 (C_8H_3NOF) ion which can be derived from either anthranilate region. Since fewer bonds need to be broken to form the 148 ion from the A2 moiety than from the A3 moiety, the fluorine was assigned to the A2 moiety when the 148 ion was intense (structure **6**). When fluorinated tryptophan derivatives were incorporated, the

Fig. 2. Diagramatic representation of the analytical HPLC of (A) the leucine-derived analogs (**17**~**28**) in system 1, (B) the tryptophan-derived analogs (**4**~**16**) in system 2, and the natural products (**1**~**3**) in both systems.



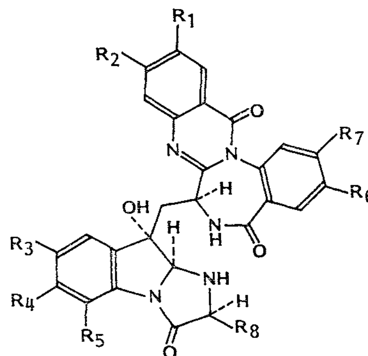
A 10-μl (whole broth equivalent) injection of a crude extract from a resting cell preparation (see Table 3) gave the indicated response at 254 nm (see Experimental section for details).

position of the fluorine in the tryptophan ring was assumed to be the same as in the parent compound. The assignments of the amino acids at R_8 in compounds **17**~**28** (Table 4) were confirmed by ^{1}H NMR.

Approximately 20 analogs of anthranilate and 20 of Phe were screened in resting cells. For the most part, the compounds contained aromatic substitutions including: CH_3, halogens, NO_2, OH, OCH_3, and NH_2. Little, if any, of these compounds were incorporated into the basic structures. Of course we would have been surprised and intrigued if Phe analogs had been converted to an asperlicin-like compound. Interestingly, *ortho*-fluorophenylalanine stimulated production of asperlicin with about 1/3 the efficacy of phenylalanine. Anthranilate analogs may not be substrates for the enzymes involved in tryptophan biosynthesis, thus preventing substitution at the Trp moiety. Considering the results with the tryptophan analogs (see below), we were surprised that anthranilate analogs did not substitute at A2 or A3. Very possibly the cells have low permeability to anthranilate.

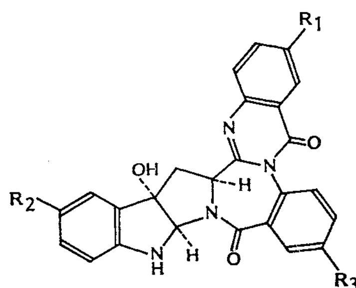
Fluoro- and methyl-substituted tryptophans were incorporated into the analogous position (indole, A1) of all three natural products (Tables 4~6). Moreover, many of the Trp derivatives were broken

Table 4. Asperlicin derived analogs.



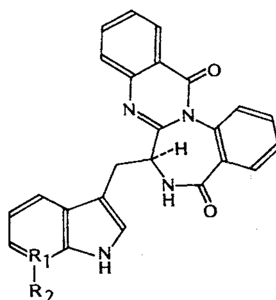
Compound	R_1	R_2	R_3	R_4	R_5	R_6	R_7	R_8	Empirical formula	Molecular ion	Fragment 1	Fragment 2
Asperlicin (**1**)	H	H	H	H	H	H	H	$CH_2CH(CH_3)_2$	$C_{31}H_{29}N_5O_4$	535	277	259
4	H	H	F	H	H	F	H	$CH_2CH(CH_3)_2$	$C_{31}H_{27}N_5O_4F_2$	571	295	277
5	F	H	F	H	H	F	H	$CH_2CH(CH_3)_2$	$C_{31}H_{26}N_5O_4F_3$	589	313	277
6	F	H	F	H	H	H	H	$CH_2CH(CH_3)_2$	$C_{31}H_{27}N_5O_4F_2$	571	295	277
10	H	H	H	H	H	H	F	$CH_2CH(CH_3)_2$	$C_{31}H_{28}N_5O_4F$	553	295	259
11	H	F	H	H	H	H	F	$CH_2CH(CH_3)_2$	$C_{31}H_{27}N_5O_4F_2$	571	313	259
12	H	F	H	F	H	H	F	$CH_2CH(CH_3)_2$	$C_{31}H_{26}N_5O_4F_3$	589	313	277
14	CH_3	H	H	H	H	CH_3	H	$CH_2CH(CH_3)_2$	$C_{33}H_{33}N_5O_4$	563	305	259
16	H	H	H	H	CH_3	H	H	$CH_2CH(CH_3)_2$	$C_{32}H_{31}N_5O_4$	549	277	273
17	H	H	H	H	H	H	H	$CH_2CH(CH_3)(CF_3)$	$C_{31}H_{26}N_5O_4F_3$	589	277	313
18	H	H	H	H	H	H	H	$CH_2CH(CH_3)(CF_3)$	$C_{31}H_{26}N_5O_4F_3$	589	277	313
19	H	H	H	H	H	H	H	$CH_2C(CH_3){=}CH_2$	$C_{31}H_{27}N_5O_4$	533	277	257
20	H	H	H	H	H	H	H	$CH_2CH_2CH_3$	$C_{30}H_{27}N_5O_4$	521	277	245
21	H	H	H	H	H	H	H	$CH_2CH{=}CH_2$	$C_{30}H_{25}N_5O_4$	519	277	243
22	H	H	H	H	H	H	H	Cyclopentyl	$C_{32}H_{29}N_5O_4$	547	277	271
23	H	H	H	H	H	H	H	$CH_2CH_2SCH_2CH_3$	$C_{31}H_{29}N_5O_4S$	567	277	291
24	H	H	H	H	H	H	H	Cyclohexyl	$C_{33}H_{31}N_5O_4$	561	277	285
25	H	H	H	H	H	H	H	Phenyl	$C_{33}H_{25}N_5O_4$	555	277	279
26	H	H	H	H	H	H	H	$CH(CH_3)CH_2CH_3$	$C_{31}H_{29}N_5O_4$	535	277	259
27	H	H	H	H	H	H	H	$CH_2CH_2CH_2CH_3$	$C_{31}H_{29}N_5O_4$	535	277	259
28	H	H	H	H	H	H	H	$CH_2CH_2SCH_3$	$C_{30}H_{27}N_5O_4S$	553	277	277

Table 5. Asperlicin E derived analogs.



Compound	R_1	R_2	R_3	Empirical formula	Molecular ion	Fragment 1	Fragment 3
Asperlicin E (**2**)	H	H	H	$C_{25}H_{18}N_4O_3$	422	275	285
7	H	F	F	$C_{25}H_{16}N_4O_3F_2$	458	293	321
8	H	F	H	$C_{25}H_{17}N_4O_3F$	440	275	303
9	F	F	F	$C_{25}H_{15}N_4O_3F_3$	476	311	321

Table 6. Asperlicin C derived analogs.



Compound	R_1	R_2	Empirical formula	Molecular ion	Fragment 1	Fragment 2
Asperlicin C (**3**)	C	H	$C_{25}H_{18}N_4O_2$	406	277	130
13	N	—	$C_{24}H_{17}N_5O_2$	407	277	131
15	C	CH_3	$C_{26}H_{20}N_4O_2$	420	277	144

down to the corresponding anthranilate analogs, which in turn replaced the anthranilate moieties. For instance, 5-fluorotryptophan was converted to three of the possible seven permutations where one or more of the aromatic rings in asperlicin contained the appropriate fluoro substitution. It is more than likely that all the possible fluoro-derivatives were produced, but that only a fraction was detected and isolated. 7-Azatryptophan produced only one compound, **13**; this was the 7-azaindole derivative of asperlicin C. Apparently the final steps for the biosynthesis of **1** and **2** could not tolerate the C to N substitution. Other ring-substituted analogs of tryptophan (containing OH, NO_2, or the larger halogens) were not substrates for biosynthesis. Side chain substituents on tryptophan also prevented any biotransformation.

Over 20 leucine analogs were incubated with *A. alliaceus* in order to screen for substitution at R_8 in asperlicin (Table 4). Of the common amino acids, only methionine and isoleucine could substitute for leucine. As one might predict, amino acids with polar side chains were not incorporated. There was a definite correlation between the ability of an amino acid to incorporate at R_8 and the size of the

lipophilic side chain. For instance, valine and α-aminobutyrate were not incorporated into the analogous structures but, α-aminopentanoate (**20**) and norleucine (**27**) did substitute for leucine. In fact, while the other leucine analogs had little effect on the titer of **1**, phenylglycine and cyclohexylglycine inhibited its production by 50 to 80%. Phenylalanine and 2-aminobicycloheptane-2-carboxylate were not substrates for R_8 substitution. Incubation of trifluoromethylleucine generated equal amounts of two epimers (**17** and **18**) because the substrate was racemic at C-4. Ethionine not only produced an analog (**23**), it selectively stimulated the production of the minor metabolites, **2** and **3** (Table 1). The side chains of allylglycine and 2-[3-cyclopentenyl]glycine were reduced in the process of being converted to **20** and **22**, respectively. This is in contrast to the production of **19** from methylallylglycine. When **21** (which is unsaturated) was incubated with resting cells, there was no evidence of further metabolism to **20** (saturated). Thus, the olefinic side chains of these amino acids may be reduced prior to the biosynthetic sequence. Considering the variety of substrates that could substitute for leucine (allyl vs. cyclohexyl), we must conclude that the biosynthetic machinery that converts **3** to asperlicin has a wide structural tolerance for the leucine derived residue. On the other hand, the enzymes that produce **3** are more specific and cannot accept more than minor substitutions (H to CH_3) on the natural precursors.

In hand we had both the microbiological protocol, and the analytical techniques for the directed biosynthesis of asperlicin analogs. First of all, the resting cell system provided a means for screening a large number of compounds under substrate-defined conditions. Thus, although it may have lacked the elegance of mutasynthesis[7], this system was infinitely more expedient because we avoided the process of an auxotroph screen and still maintained some control over specific substitutions (leucine vs. tryptophan). The sensitivity (less than 1 μg/ml) and resolution of the HPLC method were more than adequate for detection and isolation of the novel analogs. Structural identification was relatively straightforward because the MS fragmentation pathways and the NMR spectra of the natural products were well defined[3,4]. Using these procedures we have accomplished two goals; elucidation of the biosynthetic precursors of asperlicin, and the production of novel asperlicin analogs.

References

1) CHANG, R. S. L.; V. J. LOTTI, R. L. MONAGHAN, J. BIRNBAUM, E. O. STAPLEY, M. A. GOETZ, G. ALBERS-SCHÖNBERG, A. A. PATCHETT, J. M. LIESCH, O. D. HENSENS & J. P. SPRINGER: A potent nonpeptide cholecystokinin antagonist selective for peripheral tissues isolated from *Aspergillus alliaceus*. Science 230: 177~179, 1985
2) GOETZ, M. A.; M. LOPEZ, R. L. MONAGHAN, R. S. L. CHANG, V. J. LOTTI & T. B. CHEN: Asperlicin, a novel non-peptidal cholecystokinin antagonist from *Aspergillus alliaceus*. Fermentation, isolation and biological properties. J. Antibiotics 38: 1633~1637, 1985
3) LIESCH, J. M.; O. D. HENSENS, J. P. SPRINGER, R. S. L. CHANG & V. J. LOTTI: Asperlicin, a novel non-peptidal cholecystokinin antagonist from *Aspergillus alliaceus*. Structure elucidation. J. Antibiotics 38: 1638~1641, 1985
4) LIESCH, J. M.; O. D. HENSENS, D. L. ZINK & M. A. GOETZ: Novel cholecystokinin antagonists from *Aspergillus alliaceus*. II. Structure determination of asperlicins B, C, D, and E. J. Antibiotics 41: 878~881, 1988
5) NAKAGAWA, M.; M. TANIGUCHI, M. SODEOKA, M. ITO, K. YAMAGUCHI & T. HINO: Total synthesis of (+)- and (−)-tryptoquivaline G by biomimetic double cyclization. J. Am. Chem. Soc. 105: 3709~3710, 1983 and references cited therein
6) YEULET, S. E. & P. G. MANTLE: Biosynthesis of auranthine. FEMS Microbiol. Let. 41: 207~210, 1987
7) RINEHART, K. L.: Mutasynthesis of new antibiotics. Pure and Appl. Chem. 49: 1361~1384, 1977