NOVEL CHOLECYSTOKININ ANTAGONISTS FROM *ASPERGILLUS ALLIACEUS*

I. FERMENTATION, ISOLATION, AND BIOLOGICAL PROPERTIES

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The discovery and biological properties of four novel cholecystokinin antagonists produced by *Aspergillus alliaceus* is described. One of these was seven times more potent than the previously reported asperlicin.

We recently reported the discovery of asperlicin, a novel non-peptidal antagonist of the gastrointestinal hormone neurotransmitter cholecystokinin (CCK)[1~3]. Continued examination of fermentation broths produced by *Aspergillus alliaceus* has resulted in the isolation of four further CCK antagonists, all related to asperlicin, one of which is seven times more potent than the parent compound. This paper describes the fermentation, isolation, and biological properties of these novel entities, named asperlicins B, C, D, and E.

Fermentation

Cultures

Aspergillus alliaceus ATCC 20655 and 20656 were used in this study.

Fermentation Conditions

Inocula were obtained by growing the culture in 250-ml unbaffled Erlenmeyer flasks at room temperature without agitation on medium I[2] for 7 to 21 days. After incubation, flasks were agitated to disperse and wet the spores. Flask contents were used to inoculate production media (2.5% inoculum). Production media were incubated with agitation at 28°C for 4 to 7 days.

Results

The asperlicins described here are produced along with asperlicin in a wide variety of complex production media. The media listed in Table 1, for example, were part of an optimization program for the production of asperlicin itself. Asperlicins C, D, and E were isolated from these media. Asperlicin B was isolated from a similar medium that contained corn meal 5 g/liter, Ardamine pH 5 g/liter, lard water 5 g/liter, Pharmamedia 20 g/liter, Na-citrate 2 g/liter, phenylalanine 2 g/liter, tryptophan 2 g/liter, KH_2PO_4 2 g/liter, $(NH_4)_2SO_4$ 1 g/liter, and polyglycol P2000 1 ml/liter.

Isolation and Purification

Whole broth (250 ml) was vigorously shaken with an equal volume of ethyl acetate. The organic phase was concentrated under reduced pressure; the resultant oily mass was diluted to 15 ml with methylene chloride and applied onto a 200-ml bed of silica (E. Merck Silica gel 60) equilibrated with

Table 1. Media composition.

	Ardamine pH (g/liter)	KH_2PO_4 (g/liter)	$(NH_4)_2SO_4$ (g/liter)	Corn meal (g/liter)	Lard water (g/liter)	Soybean meal (g/liter)	Cod liver oil (g/liter)	Sodium citrate (g/liter)	Asperlicin titer (μg/ml)
S1	5	2	2	10	5	5	2	2	27
S2	0	5	0	25	10	0	1	0	8
S3	20	0	5	0	10	10	4	5	23
S4	20	1	2	5	10	0	0	10	18
S5	5	2	2	20	0	5	4	10	36
S6	10	2	0	0	20	20	0	5	47
S7	10	5	5	10	10	10	1	0	67
S8	5	2	1	5	5	20	2	2	83
S9	0	0	2	20	15	5	2	5	51
S10	18.0	0	4.8	0	8.8	18.8	2.8	9.8	22

Table 2. Physico-chemical properties and chromatographic behavior of asperlicins B~E.

	Asperlicin B	Asperlicin C	Asperlicin D	Asperlicin E
Appearance	Colorless powder	Off-white powder	Colorless powder	Off-white powder
MW (MS)	551	406	406	422
Molecular formula	$C_{31}H_{29}N_5O_5$	$C_{25}H_{18}N_4O_2$	$C_{25}H_{18}N_4O_2$	$C_{25}H_{18}N_4O_3$
UV λ_{max}^{MeOH} nm (ε)	228 (sh, 47,000), 256 (sh, 16,200), 265 (sh, 13,200), 280 (sh, 9,800), 310 (4,000)	222 (56,300), 268 (15,100), 278 (14,000), 289 (sh, 8,480), 310 (4,650), 321 (sh, 3,590)	222 (61,060), 283 (sh, 13,790), 290 (14,600), 310 (5,920)	213 (sh, 37,150), 227 (38,450), 268 (8,680), 277 (sh, 7,830), 310 (sh, 4,850), 324 (3,200)
Rf silica gel[a] I:	0.70	0.65	0.70	0.60
II:	0.65	0.75	0.80	0.70
k' HPLC[b]	4.9	6.5	7.7	3.0

[a] E. Merck Silica gel $60F_{254}$, I: CH_2Cl_2 - MeOH (95 : 5), II: EtOAc.
[b] Whatman ODS-3, 10 μm particles, 2.0 ml/minute, CH_3CN - H_2O(6 : 4), 40°C.

methylene chloride - methanol (97.5 : 2.5). After elution with one column volume of the same solvent mixture, the column was washed with methylene chloride - methanol (95 : 5).

Fractions containing asperlicins C, D, and E were obtained at 1.2~1.6 column volumes, while large amounts of asperlicin eluted later (2~3 column volumes). The three components of interest were separated by reverse phase liquid chromatography (Whatman ODS-3 9 mm×50 cm column packed with 10 μm particles, acetonitrile - water (40 : 60) delivered at 10 ml/minute at 40°C). E: Volume of retention 2.4 column volumes, yield 90%, C: 5.0 column volumes, yield 85%, D: 6.1 column volumes, yield 85%. The purity of the isolated compounds was ascertained in several TLC systems and by HPLC (see Table 2).

The fourth minor component (asperlicin B) produced only in very small amounts, was detected when isolation of C~E was carried out on larger scale.

The ethyl acetate extract of 320 liters of fermentation broth was concentrated to small volume, diluted with methylene chloride - methanol (3 : 1) and adsorbed onto 750 g of silica gel by stirring; after washing the adsorbent with methylene chloride to remove the non-polar impurities, the components of interest were eluted with methylene chloride - methanol (4 : 1). After evaporation of the solvent, chromatography was carried out on a 4-liter silica column equilibrated and eluted with methylene chloride - methanol (95 : 5). Early fractions (volume of retention 0.8~0.95 column volumes)

contained mainly B; components C~E, together with *ca.* one half of the asperlicin present were found in later fractions (1.0~1.4 column volumes).

Table 3. Effect of asperlicins on [^{125}I]CCK binding in rat pancreatic membranes.

	IC_{50} (μM)
Asperlicin	1.4
Asperlicin B	0.2
Asperlicin C	22
Asperlicin D	76
Asperlicin E	680

Fractions containing the crude B were first purified by silica gel column chromatography (ethyl acetate - methylene chloride (4:1)), followed by reverse phase liquid chromatography (Whatman ODS-3, 9 mm×50 cm column packed with 10 μm particles, acetonitrile - water (25:75) at 10 ml/minute, 40°C); yield of pure B 86%.

The individual components from the C~E+asperlicin fractions were conveniently separated by gel filtration (Sephadex LH-20, 2.5×200 cm column, elution with methanol). Asperlicin: 0.95 column volumes, E: 1.3 column volumes, C: 1.5 column volumes, and D: 1.6 column volumes. Final purification was achieved by: Component E: multiple recrystallizations from methanol, yield 66%, component C: reverse phase liquid chromatography (E. Merck LiChroprep RP-18 25~40 μm particles, column dimensions 1.4×183 cm, acetonitrile - water (35:65) delivered at 20 ml/minute, 40°C), yield 82%, and component D: recrystallization from ether, yield 70%.

Biological Properties

The methods employed to study displacement of [^{125}I]CCK-33 binding from rat pancreatic receptors and antagonism of CCK-induced contraction of the guinea pig gall bladder have been described previously[1].

Asperlicins B~E inhibited specific [^{125}I]CCK-33 binding in rat pancreatic membranes in a concentration-dependent manner. Their potencies in comparison to asperlicin itself are shown in Table 3. Asperlicin B (IC_{50} 0.20 μM) was 7 times more potent than asperlicin, whereas the other compounds were less potent.

The well-known action of CCK-8 in contracting the isolated guinea pig gall bladder was used to further study the interaction of asperlicin B with peripheral CCK receptors. At 0.64 μM, asperlicin B, alone, did not affect contractions, indicating a lack of agonist activity. However, at this concentration, it caused a 4.7±0.8-fold shift to the right of the dose-response curves for CCK-stimulated contraction. The maximal response to CCK-8 in the presence of asperlicin B did not differ significantly from that obtained with CCK-8 alone, indicative of competitive antagonism. The dissociation constant for asperlicin B as an antagonist of CCK in this system was 0.17 μM, which was comparable to that obtained from [^{125}I]CCK binding studies in pancreas.

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