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Chemical identity of the acrasin of the cellular slime mold *Polysphondylium violaceum*

(glorin/peptide/chemoattractant/chemotaxis/amoeba)

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ABSTRACT **The aggregation chemoattractant (or acrasin) of *Polysphondylium violaceum*, a species of cellular slime mold that does not respond chemotactically to cAMP, has been identified. It was extracted and purified from aggregating amoebae, then analyzed for amino acid composition and by IR and mass spectrometry. The active molecule is *N*-propionyl-γ-L-glutamyl-L-ornithine-δ-lactam ethyl ester (M_r, 327), which we have named *glorin*. The compound has been synthesized and shows normal chemotactic activity with the amoebae of *P. violaceum*.**

It has been known since the work of Shaffer (1, 2) that the chemoattractant or acrasin of *Polysphondylium violaceum* differs from that of the larger species of *Dictyostelium*. In 1968 (3, 4), it was shown that the acrasin of *Dictyostelium* is cAMP and that this substance, even though secreted by *Polysphondylium* amoebae, does not serve as a chemoattractant for that species (5–7).

The search for the chemical identity of *P. violaceum* began in this laboratory in 1973. A preliminary characterization was published in 1976 (8). At that time, there was evidence that it was a small molecule and it was suggested that it might be a peptide with its amino and carboxyl groups completely blocked. This early proposal has turned out to be correct.

MATERIALS AND METHODS

Glorin Collection. The basic method of collection of the *P. violaceum* acrasin used was that devised by Francis (9) for *Polysphondylium pallidum*. Late vegetative amoebae were washed and placed on nonnutrient agar. When they were in full aggregation, 40% ethanol was poured over each Petri dish to inhibit the acrasinase and this ethanol solution was collected and concentrated.

Here are the details of the process. *P. violaceum* strain 1 was grown with *Escherichia coli* strain B/r in 40 large Petri dishes (15 × 150 mm) at 22°C for 40 hr in diffuse light on buffered agar/1% peptone/1% dextrose. The amoebae were harvested in 1% standard salt solution (10) and washed three times by centrifugation (5 min at 150 × *g*). The pellets of amoebae were diluted in 50% standard salt solution to produce a suspension of 6×10^6 cells/ml. Fifteen milliliters of this suspension was placed in each of 150 large Petri dishes (15 × 150 mm) containing 2% nonnutrient agar and, after the amoebae had settled, the supernatant was carefully poured off and the plates were allowed to drain. All excess liquid was removed with filter paper and the plates were dried without covers for 10 min and then covered. After about 2 hr, when the amoebae were actively streaming into aggregations, each Petri dish was rinsed with 10 ml of 40% ethanol, which was decanted, and then rinsed again with fresh 40% ethanol (10 ml over four successive Petri dishes). The final collection (2,000 ml) was centrifuged at 16,300 × *g* for 10 min, and the supernatant boiled down to about 50 ml and stored at −15°C.

Chemotaxis Assay. Chemotaxis was tested as described (8, 11) with two modifications. First, a 2-μl drop of amoebae was placed directly on the agar instead of on a square of cellophane. If there was acrasin in the agar, the amoebae moved out more rapidly than in the control, presumably because the stable acrasinase removed the acrasin in the vicinity of the drop and the cells moved directly outward toward the higher concentration of acrasin beyond the drop. The second modification was that, instead of scoring the distance the cells moved out, the pattern of the response was scored qualitatively (Fig. 1). This visual scoring was found to be a more convenient index of high activity. *P. violaceum* amoebae are at the right stage of aggregation competence for a very restricted period of time and, immediately after they have passed their brief aggregation-sensitivity peak, the cells tend to reverse themselves and move back toward the new centers that have formed inside the original drop and begun to secrete their own glorin. Thus, the distance of outward movement from the drop is extremely variable, even in successive bioassays testing the same quantity of glorin. For this reason, we always ran two controls: (*i*) no glorin and (*ii*) a standard concentration that gave strong activity; only in this way could we be certain that the assay itself was working correctly.

Purification of Glorin. The purification procedure for 40 batches (150 Petri dishes each) of crude extract is summarized in Table 1. The extract was first filtered through a column of QAE-Sephadex (hydrochloride form) prepared with water to remove acidic impurities, which was washed with 2 column vol of water. The filtrates and washings of all the batches were combined and concentrated under reduced pressure to 30 ml. The concentrate was mixed with 200 ml of ethanol and the resulting precipitate was removed by centrifugation. The supernatant was evaporated to dryness under reduced pressure and the residue was dissolved in 50% ethanol for the column chromatography of step 2.

A Pharmacia K column with Tygon tubing and a Buchler peristaltic pump were used in steps 2–5. Step 6 was done on a Cheminert type LC column (Applied Science Division, State College, PA) with Viton tubing. In steps 7–9, an organic solvent-resistant column (Reliance Glass Works, Bensenville, IL; no. R-2790-500) with Teflon tubing was used, and solvent delivery was by gravity. In each step, fractions of the eluate were tested for chemotactic activity and the absorption spectrum (210–320 nm) was examined. Because the target compound does not absorb significantly above 210 nm, the decrease of absorption in

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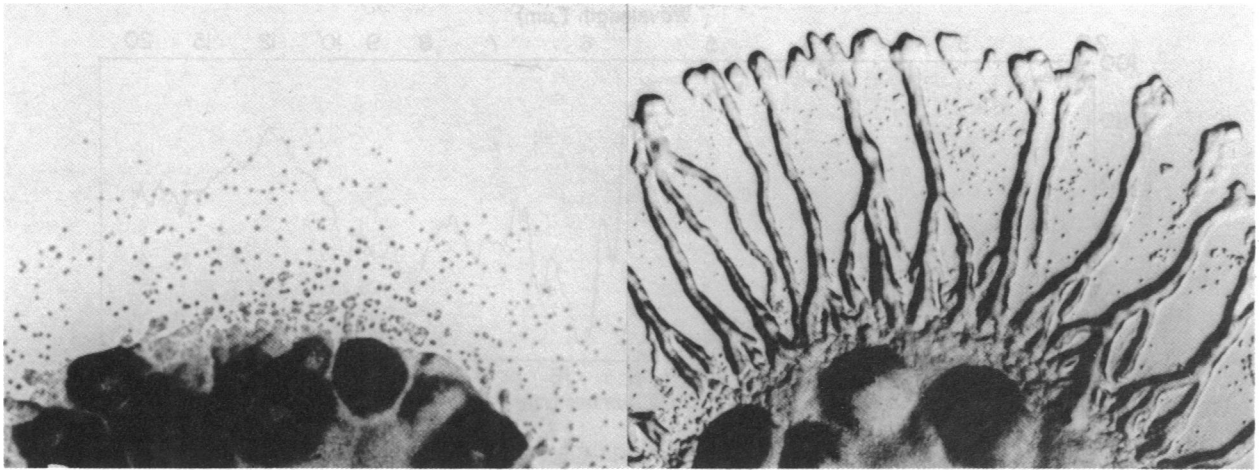

FIG. 1. Chemotaxis test for glorin. (*Left*) Control with no acrasin. (*Right*) A maximal response to acrasin in the agar; all the amoebae are incorporated into very strong streams. (This is recorded as a ++++ response; a +++ response is one in which there is a strong sunburst of streams moving out through a dense front of amoebae; ++ indicates a dense front with some streams; and a + response is one in which there are no streams, yet the amoebae are moving out in a dense front.)

the region of this wavelength at each step was a convenient indicator of the progress of purification. Thus, the values of total absorbance (A_{1cm} × total volume in ml) at 250 nm for steps 2–9 were 230, 140, 7.8, 3.7, 0.75, 0.25, and 0.07, respectively.

Absolute ethanol (Publicker Chemical, Greenwich, CT) was used without further purification for harvesting; that used in chromatography was glass distilled before use. Acetone and acetonitrile were both spectrophotometric grade (Aldrich). Amino acid analyses were carried out by Sequemat (Watertown, MA) after hydrolysis with 5.7 M HCl at 110°C for 24 hr. The IR spectrum was measured by Sadtler Research Laboratory (Philadelphia), and mass spectrometry was done at the Mass Spectrometry Facility (Massachusetts Institute of Technology).

Synthesis of Glorin. The synthesis was carried out at Peninsula Laboratories (San Carlos, CA), by Ding Chang and K. Channabasavaiah. L-Glutamic acid γ-benzyl ester was first propionated with propionic anhydride and then esterified by treatment with thionyl chloride followed by ethanol, yielding propionyl-L-glutamic acid α-ethyl-γ-benzyldiester. Treatment of the diester with HF gave propionyl-L-glutamic acid α-ethyl ester. This compound was condensed with L-ornithine-δ-lactam (12) by the mixed anhydride procedure with isobutyl chloroformate and *N*-methylmorpholine. The product was purified by column chromatography on Sephadex LH-20 using acetonitrile/water, 9:1 (vol/vol) as the eluent.

RESULTS

Identification of Glorin. Glorin has certain properties that limit the method of purification. (*i*) As reported (8), it is nonionizable. (*ii*) On silica gel columns, glorin forms a very broad band with a variety of eluents and almost 50% of the activity is

Table 1. Purification of glorin by column chromatography

Step	Conditions	Total activity (A)*	Dry weight (B), mg	Specific activity, A/B
	(Crude extracts from 40 batches)	20,000	4,000	5
1	Filtration through QAE-Sephadex, 2.5 × 10 cm, 2 columns			
2	Sephadex LH-20, 2.5 × 82 cm, 50% ethanol, 8 columns	15,000	150	100
3	Repeat of step 2, 3 columns	12,000		
4	Bio-Gel P-30 (100–200 mesh), 1.6 × 90 cm, 50% ethanol, 2 columns	10,000	6	1,670
5	Repeat of step 4, 2 columns	9,000		
6	Sephadex LH-20, 9 mm × 68 cm, 55% ethanol, 1 column	8,000		
7	Sephadex LH-20, 8 mm × 90 cm, 55% ethanol, 1 column	6,000	0.5	12,000
8	Sephadex LH-20, 8 mm × 83 cm, acetone/water, 8:2 (vol/vol), 1 column	4,500	0.220	20,500
9	Sephadex LH-20, 8 mm × 85.5 cm, acetonitrile/water, 8:1 (vol/vol), 1 column	2,500	0.092†	27,200

*Total activity is given in activity units, where 1 unit corresponds to an activity of +++ in the chemotaxis assay (see Fig. 1).

†A colorless resin.

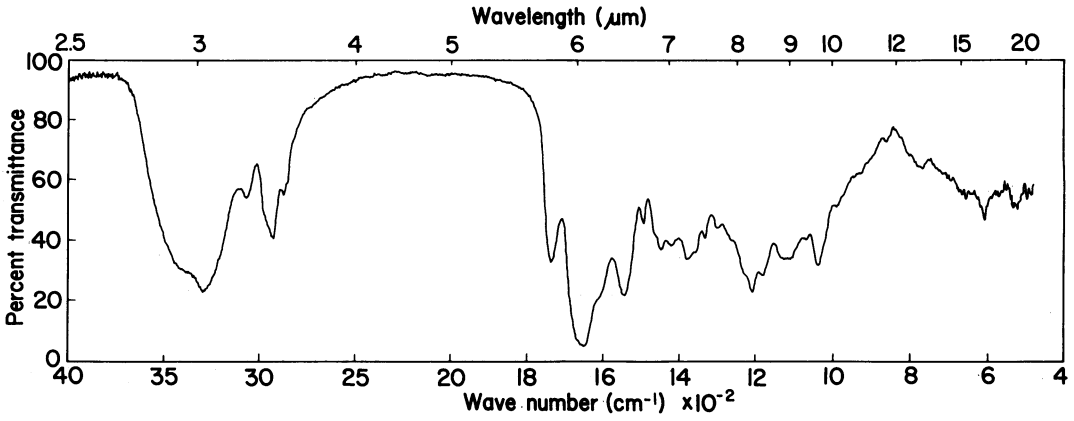

FIG. 2. Fourier transform IR spectrum (18.4 μg of glorin in a KBr micropellet).

lost. (*iii*) With Sephadex G-25 and other gels and an aqueous eluent, there is a very large loss of activity (>90%) despite the fact that glorin is stable when dissolved and left standing in water. The most successful method is the use of a Sephadex LH-20 column and an eluent that contains at least 50% organic solvent.

Purified glorin does not show an absorption peak above 210 nm in either the UV or visible region; there is some absorption at 210 nm, but it falls off steeply and becomes negligible above 260 nm. The IR spectrum resembles those of proteins and polypeptides (e.g., casein or gelatin), suggesting that glorin is probably a peptide (Fig. 2). The only significant difference is a peak at 1,739 cm^{-1} that may be due to the C=O stretching vibration of an ester group in the glorin molecule.

Amino acid analysis of 9.2 μg of purified glorin showed, after acid hydrolysis, only two amino acid residues: 29.5 nmol of glutamic acid and 27.3 nmol of ornithine. If one assumes that the molecule contains 1 mol each of these two substances, the molecular weight is calculated to be 312 (from the amount of glutamic acid) or 337 (from the amount of ornithine).

Mass spectrometry using ionization techniques of field desorption and fast-atom bombardment showed that the molecular weight of this compound is 327. Furthermore, from the high-resolution fast-atom-bombardment mass spectrum, it was evident that the elemental composition of the M+1 species is $C_{15}H_{26}N_3O_5$ (observed mass, 328.187265; calculated mass, 328.18724). Therefore, the elemental formula of glorin is $C_{15}H_{25}N_3O_5$.

The fragmentation pattern of the low-resolution electron-impact-ionization mass spectrum (Fig. 3) suggests the presence of an ethyl ester group (M−46 peak) and, from the elemental formula and the presence of the two amino acid residues, it can be deduced that there must be a propionamide group (CH_3CH_2CONH). Both of these groups were confirmed by the high-resolution mass spectral data shown in Table 2 (M−C_2H_5OH, 281.13718; M−$COOC_2H_5$, 254.15013; M−$C_2H_5CONH_3$, 253.11913).

Therefore, the molecule of glorin consists of one glutamic acid residue [$COCH_2CH_2CH(NH)CO$], one ornithine residue [$NHCH_2CH_2CH_2CH(NH)CO$], one C_2H_5O group that is bound to one of the three C=O groups of the amino acid residues, and one C_2H_5CO group that is bound to one of the three NH groups in the amino acid residues. Based on the elemental formula, the two C=O groups and two NH groups of the amino acid residues remaining unbound must exist in the form of two

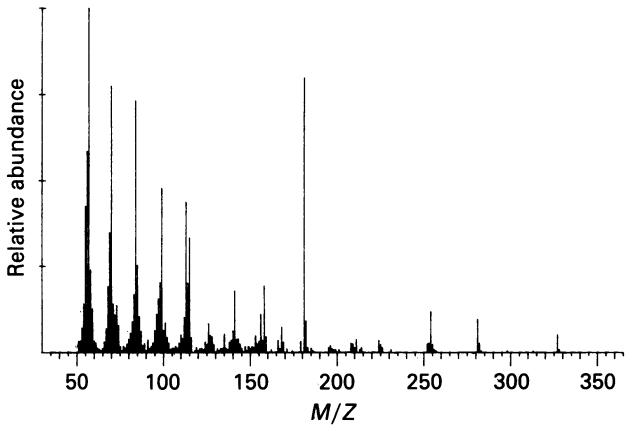

FIG. 3. Low-resolution mass spectrum of glorin by electron-impact ionization (70 eV).

Table 2. High-resolution mass spectral data of glorin obtained by electron-impact ionization

Mass		
Observed	Calculated	Elemental composition
70.06655	70.06567	C_4H_8N
84.04767	84.04494	C_4H_6NO
85.05150	85.05276	C_4H_7NO
85.07801	85.07657	$C_4H_9N_2$
98.06140	98.06059	C_5H_8NO
99.06669	99.06842	C_5H_9NO
113.04941	113.04768	$C_5H_7NO_2$
113.07318	113.07149	$C_5H_9N_2O$
114.07823	114.07932	$C_5H_{10}N_2O$
115.08814	115.08714	$C_5H_{11}N_2O$
126.08097	126.07932	$C_6H_{10}N_2O$
140.07147	140.07115	$C_7H_{10}NO_2$
141.06763	141.06640	$C_6H_9N_2O_2$
153.10260	153.10279	$C_8H_{13}N_2O$
156.08821	156.08988	$C_7H_{12}N_2O_2$
158.08079	158.08172	$C_7H_{12}NO_3$
169.09690	169.09771	$C_8H_{13}N_2O_2$
181.09880	181.09771	$C_9H_{13}N_2O_2$
196.10961	196.10860	$C_9H_{14}N_3O_2$
214.10760	214.10794	$C_{10}H_{16}NO_4$
231.13386	231.13449	$C_{10}H_{19}N_2O_4$
253.11913	253.11883	$C_{12}H_{17}N_2O_4$
254.15013	254.15047	$C_{12}H_{20}N_3O_3$
281.13718	281.13756	$C_{13}H_{19}N_3O_4$
327.17452	327.17943	$C_{15}H_{25}N_3O_5$

The most intense peaks of mass >70 are listed. Resolution: M/ΔM, 12,000.

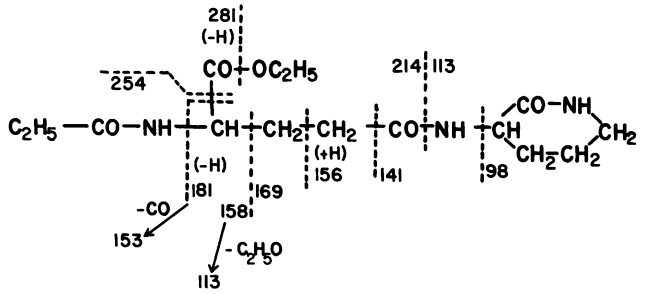

FIG. 4. Chemical structure of glorin. The major electron-impact-ionization mass spectral fragmentations are indicated.

peptide bonds—i.e., two NHCO groups.

It is possible to write 12 different structures knowing the above information. However, only one of those structures can satisfactorily explain the formation of the two fragments, 214.10760 ($C_{10}H_{16}NO_4$) and 169.09690 ($C_8H_{13}N_2O_2$). This structure (shown with some of its main fragmentations in Fig. 4) is wholly consistent with the high-resolution mass spectral data.

Biological Activity of Glorin. The substance shown in Fig. 4 was then synthesized from L-glutamic acid and L-ornithine, giving a colorless crystalline powder. Its mass spectrum was identical to the one obtained from purified natural glorin (Fig. 3).

Both the purified and the synthetic glorin were compared on the chemotaxis assay, this time using the quantitative test (11) in addition to the visual one that had been so useful in the purification (Fig. 1). Both the synthetic and the natural preparations of glorin showed identical activity with a peak at 10 nM, considerable activity at 100 nM, less at 1 nM, and none at 1 μM. This is about 10 times the cAMP activity for *Dictyostelium discoideum*, where the activity peak using the same chemotactic test is 100 nM (13).

DISCUSSION

Thus far, it is known that there are at least eight different acrasins in the cellular slime molds (14) but only one of these, cAMP, has been identified chemically. Folic acid and related substances serve as powerful attractants (15, 16) but they seem to act most effectively on vegetative amoebae rather than as an aggregation acrasin (13, 15). Recently, Konijn and co-workers (17) have shown that the acrasin of *Dictyostelium lacteum* is a pterin but not folic acid. Therefore, the structure of glorin reported here represents a different type of acrasin that has been fully identified.

There are a number of reasons why this discovery holds considerable promise. In the first place, the substance is a peptide. Like so many of the peptide attractants of leukocytes, it is also terminally blocked (18). The *N*-formylmethionyl peptides used for leukocyte chemotaxis do not attract *P. violaceum* amoebae (unpublished results), but the basic molecular similarity is striking despite the difference in specificity. Second, it must be remembered that, in the basic patterns of their life histories, the large *Dictyostelium* species that use cAMP for their acrasin are similar to *Polysphondylium*, which uses glorin. (*P. pallidum* is responsive to glorin and is presumed to have it as its acrasin, but this must be carefully checked.) This means that two entirely different signalling systems, including different acrasinases, receptor proteins, and so forth, do essentially the same thing and, therefore, the opportunity to do comparative signal–receptor physiology may be as rewarding as it has been with different neurotransmitters. The third point of interest is that *Polysphondylium* also secretes cAMP (5, 6) which is known to be involved in differentiation, as is well established to be the case in *Dictyostelium* (refs. 19 and 20; for review, see ref. 21). Does this mean that in *Dictyostelium* cAMP performs two separate functions (and perhaps more) while in *Polysphondylium* the chemotactic system is chemically quite separate from the differentiation system? And finally, what is the relationship between glorin, cAMP, cGMP, the D factor of Hanna and Cox (22), and other key substances involved in development, including NH_3 (23) and possibly differentiation-inducing factor, the stalk-forming morphogen of Town and Stanford (24). It is hoped that the study of glorin and its activities will open up new avenues of research in our attempt to understand development in molecular terms.

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