

4-Hydroxyphenethyl alcohol — a new cytokinin-like substance from the phototrophic purple bacterium *Rhodospirillum rubrum* 1R

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Received 9 February 1995; revised version received 6 April 1995

Abstract Three compounds with cytokinin activity have been isolated from the medium of *Rhodospirillum rubrum* grown photosynthetically. Two N-6 aminopurine cytokinins revealed in the medium were identical with those obtained from *R. rubrum* cells previously. The third compound with cytokinin activity in *Amaranthus caudatus* bioassay proved to be a simple phenolic compound with elemental composition C₈H₁₀O₂. This cytokinin-like substance according to absorption spectra, mass spectrometry and ¹H NMR spectra data was identified as 4-hydroxyphenethyl alcohol.

Key words: Cytokinin-like substance; Phytohormone; Phenethyl alcohol, 4-hydroxy; Phenolic compound; *Rhodospirillum rubrum*

1. Introduction

Phytohormones, such as cytokinins, are purines with substituent attached to the N-6 position of the adenine ring. The biological activity of these cytokinins varies considerably with the length of the side-chain, degree of its unsaturation [1] and modification of the adenine moiety [2]. Certain nonpurine compounds, both synthetic and natural ones, exhibit cytokinin-like activity as well [3].

It is known that cytokinins are associated with a variety of organisms besides plants. Plant symbiotic or pathogenic bacteria, for example, as well as the phototrophic microorganisms with the oxygenic type of photosynthesis, also contain this type of phytohormones. The ability of phototrophic bacteria with anoxygenic type of photosynthesis to synthesize the purine cytokinins and accumulate them in the cells has been shown only recently on the example of the *Rhodospirillaceae* member, *R. rubrum* [4].

Here we present evidence indicating the ability of *R. rubrum* to efflux some of the purine cytokinins synthesized by the cells as well as the cytokinin-like substance attributed to simple phenolic compounds and identified as 4-hydroxyphenethyl alcohol.

2. Materials and methods

2.1. Object and conditions of cultivation

The *R. rubrum* bacterium, strain 1R, was kindly supplied by the Department of Microbiology, Moscow State University (Moscow, Russia). The cells were grown in 1 litre flasks in Ormerod's medium [5] with 0.125% (NH₄)₂SO₄ and 0.01 mg/l of biotin at *T* = 30°C, pH 6.8,

2000 lux and harvested at the stationary growth phase by centrifugation (10,000 × *g*, 10 min).

2.2. Extraction of cytokinins

The supernatant was adjusted to pH 7.6–7.8 with 1 N NaOH and the cytokinins were extracted with watersaturated (1:1) *n*-butanol. The residue after evaporation of the butanolic extract was dissolved in 96°C ethanol and the crude fraction of cytokinins was obtained.

2.3. Thin-layer chromatography (TLC)

The crude fraction of cytokinins was separated using TLC on Silufol plate in a solvent system of isopropanol/benzene/ammonia (4:1:1, v/v). Individual bands were scraped and cytokinins were extracted with 96°C ethanol.

2.4. High-performance liquid chromatography (HPLC)

Purification of individual band with *R_f* 0.8 by HPLC method on automatic system Pharmacia LKB Biotechnology (Sweden) was performed. The sample in 10% ethanol developed on Sepharon SGX C18 2.8 × 14 mm column (Czech Republic), monitored at 254 nm, collected at 11.7 min.

2.5. Spectral measurements

The UV-spectra were recorded on the Specord M-40 (Germany) and the Shimadzu UV-160 (Japan) instruments and the fluorescence spectra on the Hitachi 850 (Japan) one. The electron-impact mass spectrum (MS) was determined on a high resolution Finnigan MAT 8430 mass spectrometer. ¹H NMR spectrum (400 MHz, D₂O) was obtained on a high resolution WM 400 Bruker spectrometer, at *T* = 307 K, with DSS (sodium-4,4-dimethyl-4-silapentane sulphonate) as standard.

2.6. Cytokinin bioassays

The activity of individual cytokinins was tested using the *Amaranthus caudatus* bioassay [4]. Benzyladenine (Fluka), zeatinriboside (Sigma), (+)-1-phenyl-1,2-ethanediol (Aldrich), 2- and 4-hydroxyphenethyl alcohol (Aldrich), 3-hydroxybenzoic acid (Aldrich) were used as standards.

3. Results

3.1. Spectral characteristics and biological activity of

R. rubrum exometabolites

TLC-analysis of the crude fraction of cytokinins from *R. rubrum* medium revealed three UV-absorbing zones on silufol plate. Two of them with *R_f* 0.36 and 0.53 (zeatinriboside) by their physico-chemical properties turned out to be analogs of cytokinins from *R. rubrum* biomass described recently [4]. The third UV-absorbing band with *R_f* 0.80 revealed also high cytokinin activity in the *Amaranthus caudatus* bioassay comparable with zeatinriboside (Table 1), but its absorption spectrum was not typical for aminopurines. Two peaks at 225 nm and 277 nm as well as a shoulder at 285 nm were seen in the absorption spectrum of this cytokinin-like substance (Table 2). Under alkaline conditions the shift of these peaks up to 241 and 296 nm, respectively, were observed. Accordingly, the absorbance at 277 nm increased.

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The fluorescence spectra of the purine cytokinins and cytokinin-like substance with R_f 0.80 differed in the excitation and emission maxima as well as in the fluorescence level which was an order higher in the cytokinin-like substance (Table 3).

A blue-green colour of the R_f 0.80 spot was observed on silufol plate under spraying with 1% FeCl_3 in 96°C ethanol.

3.2. ^1H NMR spectrum of the cytokinin-like *R. rubrum* substance with R_f 0.80

The PMR spectrum of cytokinin-like substance with R_f 0.8 exhibited the following chemical shift and coupling constants: σ , ppm (J, Hz) — 7.18 (d, 2H Cytokinin bioassays. — arom., 8.1), 6.88 (d, 2H — arom., 8.1), 3.79 (t, CH_2 , 6.6) and 2.79 (t, CH_2 , 6.6).

3.3. Mass spectral characteristics of the cytokinin-like *R. rubrum* substance with R_f 0.80

The mass spectrum of the substance with R_f 0.80 showed an intense molecular ion at m/z 138 (34%) with an elemental composition of $\text{C}_8\text{H}_{10}\text{O}_2$ as 138.076 determined by accurate mass measurement. The loss of CH_3O group gives rise to the most abundant ion at m/z 107 (100%, $\text{C}_7\text{H}_7\text{O}$, 107.058). The latter undergoes further fragmentation by loss of a formyl radical to give phenyl ion. According to the results obtained from mass spectral analysis, the cytokinin-like compound isolated from *R. rubrum* could correspond to one of the following chemical structures: 2-, 3- or 4- $\text{HO}(\text{C}_6\text{H}_4)\text{CH}_2\text{CH}_2\text{OH}$ (hydroxyphenethyl alcohol) or $(\text{C}_6\text{H}_5)\text{CHOHCH}_2\text{OH}$ (1-phenyl-1,2-ethanediol). Spectral characteristics and biological activity of the structural analogs of the cytokinin-like *R. rubrum* substance with R_f 0.80.

UV-spectra of the cytokinin-like *R. rubrum* substance with R_f 0.80 in 96% ethanol and 0.01 N KOH solution differ drastically from those of 1-phenyl-1,2-ethanediol (Table 2). Typical absorption bands (209 and 257 nm) for benzene chromophore were found in the spectrum of 1-phenyl-1,2-ethanediol. Furthermore, due to the absence of the auxochrome substitution (the phenolic hydroxy group) no changes occurred in alkaline conditions.

Thus, we assumed that the structural analog of the cytokinin-like compound with R_f 0.80 would be represented by 2-, 3- or 4-hydroxyphenethyl alcohol. The spectrum character of the 3-substituted derivative (as an example, the 3-OH-benzoic acid has been taken for comparison) differs greatly from that of the compound with R_f 0.80. As seen from Table 2, a bathochromic shift of the absorption bands at 225 and 277 nm by 16–19 nm and a simultaneous increase of the ratio of the optical density values (A) of the major absorption bands, A_{242}/A_{296} , were found in the UV-spectra of the cytokinin-like *R. rubrum* compound and 4-OH-phenethyl alcohol in alkaline conditions. And, vice

Table 1
Biological activities of natural cytokinins from *Rhodospirillum rubrum* and standards

Samples	Activity in bioassay (%)
Control*	100
Natural cytokinins	
Non-purine cytokinin with R_f 0.80	350
Purine cytokinins with R_f 0.36	300
with R_f 0.53 (zeatinriboside)	330
Standards	
Benzyladenine	280
1-Phenyl-1,2-ethanediol	abs
2-OH-phenethyl alcohol	abs
4-OH-phenethyl alcohol	175

**Amaranthus caudatus* seedlings without the addition of cytokinins were used as control.

versa, a decrease of the ratio A_{241}/A_{292} was characteristic for the UV-spectrum of 2-OH-phenethyl alcohol. Thus, on the grounds of the data obtained, the cytokinin-like substance with R_f 0.8 from the *R. rubrum* medium was identified as 4-hydroxyphenethyl alcohol.

Our studies of cytokinin activity of commercial phenolic compounds in the *Amaranthus caudatus* bioassay have shown that the cytokinin-like *R. rubrum* substance with R_f 0.80 and 4-OH-phenethyl alcohol had the highest biological activity. Reliable data on such activity in other preparations have not been obtained (Table 1).

4. Discussion

The ability of *R. rubrum* to synthesize the cytokinins both of the purine line and phenolic one and to efflux them in the *R. rubrum* medium is shown in the present work. Only two purine cytokinins with R_f 0.36 and 0.53 were detected in the *R. rubrum* medium. Third purine cytokinin (R_f 0.44) revealed in the *R. rubrum* cells previously [4] was not found there.

Non-purine compound according to ^1H NMR and mass spectrometry data, the absorption spectra characters under alcohol and alkaline conditions [6,7] as well by specific staining on the chromatogram [8] was attributed to the class of simple phenolic compounds and identified as 4-hydroxyphenethyl alcohol.

It is known that some heterotrophic bacteria [9] and phototrophic purple bacterium *R. rubrum* [10] can synthesize simple phenolic compounds. The cytokinin-like compound isolated by us from *R. rubrum* can be a derivative of 4-hydroxyphenylacetic acid revealed in *R. rubrum* previously [10].

From the cytokinin activity data of the 4-hydroxyphenethyl

Table 2
Ultraviolet spectra of cytokinin-like *R. rubrum* compound with R_f 0.80 and standards

Samples	Absorption maxima (EtOH; nm)	Absorption maxima (0.01 N KOH; nm)
(1) Cytokinin-like with R_f 0.80	225, 277, 285*	241, 296
(2) 4-OH-phenethyl alcohol	225, 278, 285*	242, 296
(3) 2-OH-phenethyl alcohol	215, 274, 280*	241, 292
(4) 3-OH-benzoic acid	208, 232, 297	210, 243*, 314
(5) 1-phenyl-1,2-ethanediol	209, 252, 257, 262	216, 252, 257, 262

*Shoulder.

Table 3
Levels and maxima of fluorescence excitation and emission of natural cytokinins and standards

Samples	Excitation maximum (r.u.)	Level of excitation (nm)	Emission maximum (nm)	Level of emission (r.u.)
Natural cytokinins				
Non-purine with R_f 0.80	230	2.8	306	17
	245	1.5	306	1.7
	280	12.00	306	120.00
Purine with R_f 0.53	240	1.25	390	10.80
	280	0.58	306	2.60
Standards				
2-OH-phenethyl alcohol	260	11.8	302	116.00
	280	14.2	310	116.00
4-OH-phenethyl alcohol	245	7.0	305	24.50
	280	16.5	306	140.00
1-Phenyl-1,2-ethanediol	245	6.8	360	7.50
	285	1.8	420	1.7

alcohol exhibited in the *Amaranthus caudatus* bioassay, specific for this class of phytohormones, it was turned out to cytokinin-like compound. A necessary condition for manifestation of its biological activity in this bioassay was the arrangement of the hydroxyl group in the aromatic ring namely in the 4-position. The hydroxyl groups in phenolic compounds are known to be major functional groups specifying their chemical activity. In particular, just these groups participate in binding with proteins [11]. On this assumption one may suggest that the activity in the *Amaranthus caudatus* bioassay of the nonpurine *R. rubrum* cytokinin-like compound is as specific as for purine cytokinins and results from interaction with the protein receptor.

The formation of purine and phenolic cytokinins by other members of purple and green sulphur phototrophic bacteria as well as their action in bacterial cells and plants will be the subject of further investigations.

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