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Physarigins A–C, three new yellow pigments from a cultured myxomycete *Physarum rigidum*

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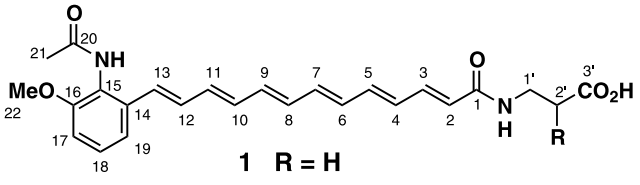
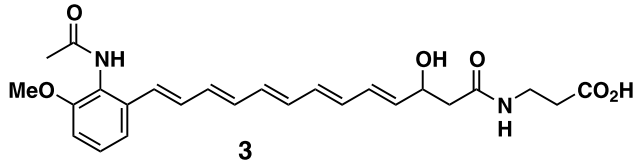
Abstract—Physarigins A–C (**1**–**3**), three new pigments have been isolated from a cultured plasmodium of myxomycete *Physarum rigidum* and their structures were elucidated by spectral data.

The myxomycetes (true slime molds) are an unusual group of primitive organisms that may be assigned to one of the lowest classes of eukaryotes. During our studies on the search for natural products from myxomycetes,[1,2] we recently investigated laboratory culture of myxomycetes and isolated sterols and pyrroloiminoquinone pigments.[3,4] Studies on the constituents of cultured myxomycetes have been very limited except for only one species *Physarum polycephalum*, from which isolation of several pigments[5–9] or bioactive lysophosphatidic acid[10] had been described. Recently we studied spore germination experiments of hundreds of field-collected myxomycetes collected in Japan, and succeeded in laboratory culture of plasmodia of several myxomycetes in a practical scale for natural products chemistry studies.[1,3,4] Here we describe the isolation and structure elucidation of three new pigments, physarigins A–C (**1**–**3**) from the cultured plasmodium of the myxomycete *Physarum rigidum*.





The fruit bodies of the myxomycetes *P. rigidum* were collected at Tokorozawa, Saitama Prefecture, Japan, in June, 2001. The plasmodium of this myxomycete obtained in a plate culture was mass cultured in the laboratory by agar plates with oatmeal according to the methods described previously.[3,4] The harvested plasmodial cells (32.4 g from 1050 plates (9 cmϕ)) were extracted with 90% MeOH and 90% acetone, and the combined extract (4.7 g) was partitioned between hexane and 90% MeOH. The 90% MeOH layer, which was revealed to contain a complex mixture of yellow pigments by TLC examinations, was subjected to ODS column chromatography (column A; 0–100% MeOH in H_2O), and the fractions eluting with 80% MeOH, containing major yellow pigments, were further separated by the flash chromatography on ODS [60% MeOH with 0.1% trifluoroacetic acid (TFA)], followed by purification with silica column ($CHCl_3$/MeOH/H_2O, 8:2:1) to give two yellow pigments, physarigins A (**1**) and B (**2**) in 0.05 and 0.006% yield, respectively. On the other hand, another fraction of column A eluting with 50% MeOH, which also mainly contained mixture of yellow pigments, was separated by silica column ($CHCl_3$/MeOH/H_2O, 7:3:1) to afford third yellow pigment, physarigin C (**3**) in 0.1% yield.

Keywords: myxomycete; *Physarum rigidum*; pigment; culture; plasmodium.

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Physarigin A (**1**) was obtained as a yellow amorphous solid, and showed absorption maxima at λ_{max} (MeOH) 403 (ε 26000) and 426 nm (22000), indicating the presence of a conjugated system. The positive and negative ESIMS spectra of **1** showed quasi-molecular ion peaks at m/z 459 $(M+Na)^+$ and at m/z 435 $(M-H)^-$, respectively, and its molecular formula was suggested as $C_{25}H_{28}O_5N_2$ by its positive HRESIMS data [m/z 459.1907, $(M+Na)^+$, Δ +1.1 mmu]. The ^1H NMR spectrum of **1** in CD_3OD (Table 1) showed signals due to two singlet methyls, two sp^3 methylenes [δ_H 3.49 (2H, t, $J=6.8$ Hz) and 2.47 (2H, t, $J=6.8$ Hz)], and many sp^2 (olefinic or aromatic) protons. The chemical shifts of the two singlet methyls [δ_H 2.17 (3H, s) and 3.81 (3H, s)] implied that these two methyl groups were acetyl and methoxy groups, respectively. The ^{13}C NMR spectrum aided by consideration of the molecular formula revealed the presence of eighteen sp^2 carbons, two amide (δ_C 167.5 and 172.0) and one acid moieties (δ_C 176.0), thus accounting for 12 out of 13 unsaturations. The remaining one was ascribable to one ring. Analysis of the 2D NMR data of **1** showed the presence of a 1,2,3-trisubstituted benzene ring [δ_H 6.92 (dd, $J=8.0$ and 1.1 Hz; H-17), 7.25 (t, $J=8.0$ Hz; H-18), and 7.27 (dd, $J=8.0$ and 1.1 Hz; H-19); ^1H–^1H COSY cross peaks: H-17/H-18 and H-18/H-19; HMBC correlations: H-17/C-15, H-17/C-19, H-18/C-14, H-18/C-16, H-19/C-17, H-19/C-15, and H-19/C-18], to which a methoxy, an acetamide, and a polyene side-chain residues were attached on C-16, C-15, and C-14 positions, respectively [δ_C 156.0 (C-16), 123.5 (C-15), and 136.0 (C-14); HMBC correlations: H_3-22/C-16, H-13/C-15, and H-13/C-19]. The polyene side-chain residue on C-14 was revealed as a tridecahexaenoyl group connected to a β-alanine unit [δ_H 3.49 (2H, t, $J=6.8$ Hz; H_2-1′) and 2.47 (2H, t, $J=6.8$ Hz; H_2-2′)] through an amide bond [δ_C 167.5 (C-1), 36.0 (C-1′), 35.0 (C-2′), and 176.0 (C-3′); HMBC correlations: H_2-1′/C-1, H_2-1′/C-2′, H_2-1′/C-3′, H_2-2′/C-1′, and H_2-1′/C-3′]. These observations and spectral data (Table 1) of physarigin A are in complete agreement with structure **1**.

Physarigin B (**2**), yellow amorphous solid; $[\alpha]_D^{22}$ −6.8 (*c* 0.5, MeOH); λ_{max} (MeOH) 403 (ε 23000) and 426 nm (20000), was suggested to have the molecular formula of $C_{26}H_{28}N_2O_5$ from the observation of a quasi-molecular ion $(M-H)^-$ at m/z 449 in its negative ESI mass spectrum and the ^{13}C NMR data aided with HMQC spectrum. The ^1H–^1H COSY and HMBC spectra of **2** suggested the presence of the same partial structures as **1** such as a 1,2,3-trisubstituted benzene ring, an acetylamino group, a methoxy group, and a dodecahexaene unit. In place of β-alanine moiety of **1**, the ^1H NMR spectrum of **2** showed one secondary methyl [δ_H 1.16 (3H, d, $J=7.2$ Hz); H_3-4′], one sp^3 methine [δ_H 2.65 (1H, m); H-2′], and one sp^3 methylene groups [δ_H 3.35 (1H, dd, $J=13.5$ and 6.0 Hz) and 3.43 (1H, dd, $J=13.5$ and 7.2 Hz); H_2-1′]. The secondary methyl protons (H_3-4′) showed HMBC correlations to the methylene (δ_C 42.0, C-1′), methine (δ_C 40.0,

Table 1. ^1H and ^{13}C NMR spectral data of physarigins A–C (**1**–**3**) in CD_3OD

Position	1			2		3	
	δ_H (J in Hz)	δ_C	HMBC (^{13}C)	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C
1	–	167.5		–	168.0	–	172.0
2	6.00 d (15.1)	123.5	C-1, C-4	6.02 d (15.0)	123.5	2.35 dd (14.1, 5.8) 2.40 dd (14.1, 8.0)	43.5
3	7.19 dd (15.1, 11.5)	141.0	C-1	7.20 dd (15.0, 11.4)	141.0	4.51 ddd (8.0, 6.6, 5.8)	68.9
4	6.40 m	130.5		6.40 m	130.5	5.73 dd (15.1, 6.6)	135.0
5	6.65 m		C-3	6.65 m	140.0	6.33 m	130.6
6	6.40 m	133.0		6.40 m	133.0	6.40 m	132.4*
7	6.50 m	133.5		6.50 m	133.5	6.40 m	133.3*
8	6.50 m	133.5		6.50 m	133.5	6.40 m	133.3*
9	6.50 m	133.5		6.50 m	133.5	6.40 m	133.4*
10	6.50 m	133.5		6.50 m	133.5	6.40 m	133.5*
11	6.50 m	133.5		6.50 m	133.5	6.50 m	134.1*
12	6.92 m			6.91 m		6.91 m	131.0
13	6.66 d (15.1)	128.5	C-11, C-15, C-19	6.66 d (15.4)	128.5	6.62 d (15.4)	127.6
14	–	136.0		–	136.0	–	136.0
15	–	123.5		–	123.5	–	123.1
16	–	156.0		–	156.0	–	155.5
17	6.92 dd (8.0, 1.1)	110.5	C-15, C-19	6.91 br d (7.8)	110.5	6.91 br d (8.1)	110.0
18	7.25 t (8.0)	128.0	C-14, C-16	7.25 t (7.8)	128.0	7.23 t (8.1)	127.8
19	7.27 dd (8.0, 1.1)	117.0	C-15, C-17, C-18	7.27 br d (7.8)	117.0	7.26 dd (8.1, 1.8)	116.9
20	–	172.0		–	172.0	–	171.7
21	2.17 (3H) s	21.5	C-20	2.17 (3H) s	21.5	2.15 (3H) s	21.2
22	3.81 (3H) s	55.0	C-16	3.81 (3H) s	55.0	3.81 (3H) s	54.9
1′	3.49 (2H) t (6.8)	36.0	C-1, C-2′, C-3′	3.35 dd (13.5, 6.0) 3.43 dd (13.5, 7.2)	42.0	3.42 (2H) m	35.1
2′	2.47 (2H) t (6.8)	35.0	C-1′, C-3′	2.65 m	40.0	2.48 (2H) t (7.5)	33.7
3′	–	176.0		–	178.0	–	174.5
4′				1.16 (3H) d (7.2)	17.5		

* Interchangeable signals.

C-2′), and carboxyl carbons (δ_C 178.0, C-3′). From these results, physarigin B (**2**) was revealed to possess a 3-aminoisobutyric acid residue instead of the β-alanine residue of **1**.[11]

Physarigin C (**3**), yellow amorphous solid; $[\alpha]_D^{22}$ +15 (*c* 1.2, MeOH); λ_{max} (MeOH) 351 (ε 9200), 368 (12000), and 390 nm (10000), exhibited a quasi-molecular ion $(M-H)^-$ at *m*/*z* 453 in its negative ESIMS, which corresponded to the molecular formula of $C_{25}H_{28}N_2O_6$, having one H_2O more than that of physarigin A (**1**). The ^1H and ^{13}C NMR data together with the ^1H–^1H COSY and HMBC experiments suggested that physarigin C (**3**) also possessed the 2-methoxyphenylacetamide and β-alanine moieties. In the ^1H NMR data, the doublet signal δ_H 6.00 (H-2) and a double doublet of low-field resonance (δ_H 7.20, H-3) observed for **1** disappeared in the ^1H NMR spectrum of **3**. Instead of them, the ^1H NMR spectrum of **3** showed signals due to an sp^3 methylene [δ_H 2.35 (1H, dd, J=14.1 and 5.8 Hz) and 2.40 (1H, dd, J=14.1 and 8.0 Hz); H_2-2] and an oxymethine [δ_H 4.51 (1H, ddd, J=8.0, 6.6, and 5.8 Hz); H-3] groups. The ^1H NMR spectrum of **3** also showed a complex of olefin proton signals assignable to a pentaene unit (δ_H 5.73–6.91, H-4 to H-13). From these observations, physarigin C was shown to have a β-hydroxy amide moiety at C-1 to C-3 position.[11]

These yellow pigments of plasmodium of *P. rigidum* are relatively unstable and not easily dissolved in organic solvents. The HPLC analysis of physarigin A (**1**) and C (**3**) [Develosil ODS-UG-5; flow rate: 1.8 mL/min; eluent: 60% MeOH with 0.1% TFA; photodiode array detection] revealed that physarigin A (**1**, t_R 32.1 min; λ_{max} 351, 368, and 390 nm) was produced from physarigin C (**3**, t_R 18.3 min; λ_{max} 426 and 403 nm) after ODS flash column chromatography using 60% MeOH with 0.1% TFA. We could not isolate a β-hydroxy amide with 2-aminoisobutyric acid moiety which may correspond to a precursor of physarigin B (**2**).

Steglich and Steffan's group previously studied plasmodial yellow pigments of *P. polycephalum*, which were considered to act as photoreceptors, and they cultured the plasmodia on oat flakes and harvested after 3 days, and isolated physarochrome A[5] and other yellow pigments[6–9] from the methanol extracts of the plasmodia. They also isolated a yellow pigment fuligorubin A[12] from field-collected plasmodium of *Fuligo septica*, and this pigment was thought to be involved in photoreceptor and energy conversion processes during the lifecycle of *F. septica*.[12] Yellow pigments of cultured myxomycetes except those of *Physarum polycepharum* had never been described before, while physarigins A–C (**1**–**3**) showed some structural similarities to physarochrome A and fuligorubin A. Compounds **1**–**3** contained a β-amino acid residue in the side chain, which was a unique structural feature and different from physarochrome A and fuligorubin A.

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11. Unfortunately, the absolute configurations of the 3-aminoisobutyric acid residue of **2** and the C-3 position of **3** remained undefined, since chiral authentic 3-aminoisobutyric acids are not available presently, and treatment of **3** with MTPA-Cl in pyridine afforded many spots on TLC examination. The geometries of double-bonds of compounds **1**–**3** were assumed as all-*E* from the similarities of the ^1H and ^{13}C NMR spectral data of phsarochrome A.[5]
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