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STRUCTURE OF TERNATIN B1, A PENTAACYLATED ANTHOCYANIN SUBSTITUTED ON THE B-RING ASYMMETRICALLY WITH TWO LONG CHAINS#

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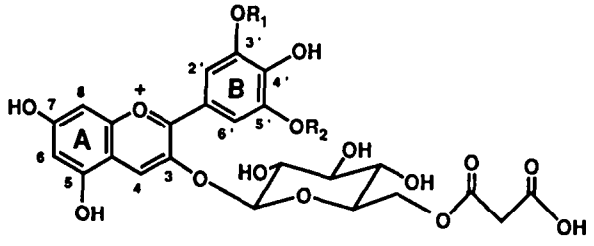
Structure of ternatin B1 isolated from blue flowers of Clitoria ternatea was determined to be 3-O-(6-O-malonyl-β-D-glucopyranosyl)-3'-O-GPGPG-5'-O-PGPG-delphinidin (1), where P = E-p-coumaryl and G = β-D-glucopyranosyl.

Saito et al.[1] isolated six highly acylated anthocyanins (ternatin A1, A2, B1, B2, D1, and D2) from the blue flowers of Clitoria ternatea. These pigments are extraordinarily stable in neutral aqueous solutions compared with other polyacylated anthocyanins. Recently Terahara et al. determined the structure of two of these anthocyanins, ternatin A1[2] and D1,[3] which have the B-ring substituted symmetrically at 3' and 5' positions.



Ternatin A1: $R_1 = R_2$ = GPGPG
Ternatin D1: $R_1 = R_2$ = PGPG
Ternatin B1: $R_1 \neq R_2$
P = E-p-Coumaryl
G = β-D-glucopyranosyl

We have also isolated three anthocyanins from the same plant. One of these, ternatin B1, showed a very complex ^{1}H NMR spectrum, indicating an asymmetrical substitution pattern, in which simple assignment of the signals as those of ternatin A1 and D1 was not possible. In this paper, we report a complete structure determination of ternatin B1 by using HMBC technique in addition to the ^{1}H-^{1}H COSY, HOHAHA, and negative difference NOE analyses.

Press juice of fresh petals of Clitoria ternatea was acidified with trifluoroacetic acid (TFA) (1.5 v/v%) and poured on a column of Amberlite XAD-7. After washing with 0.5% TFA, the column was eluted with aqueous acetonitrile (stepwise with increasing acetonitrile concentration) containing 0.5% TFA and the fractions of pigments were collected and concentrated in vacuo. Further preparative HPLC of the fractions with an ODS column

Dedicated to Professor W. D. Ollis, FRS on the occasion of his 65th birthday.

afforded pure ternatin B1, D1, and D2 as dark red TFA salt.

Ternatin B1 gave peaks at m/z 1968 (M - H + Na), 1946 (M),[4] and 1697 on its FABMS spectrum, indicating that the mass number of ternatin B1 cation is 1945. The peak at m/z 1697 (M - malonyl-Glc) suggested the presence of a malonylglucose unit in the molecule.

^{1}H NMR of ternatin B1 (Table 1) gave four doublets [α-H signals of P1 - P4 (P = p-coumaric acid); J = 16 Hz] between δ 6.2 and 5.7 ppm. In the ^{1}H-^{1}H COSY (not shown) were found the corresponding four doublets (β-H of P1 - P4; J = 16 Hz) between 7.4 and 7.0 ppm. In the difference NOE measurement (Fig. 1), irradiation at each of the four signals (α-H of P1 - P4) gave NOE on a two-proton signal of one of aromatic rings (2 and 6 positions of a p-coumaric acid). In turn, the two-proton signal had a spin coupling

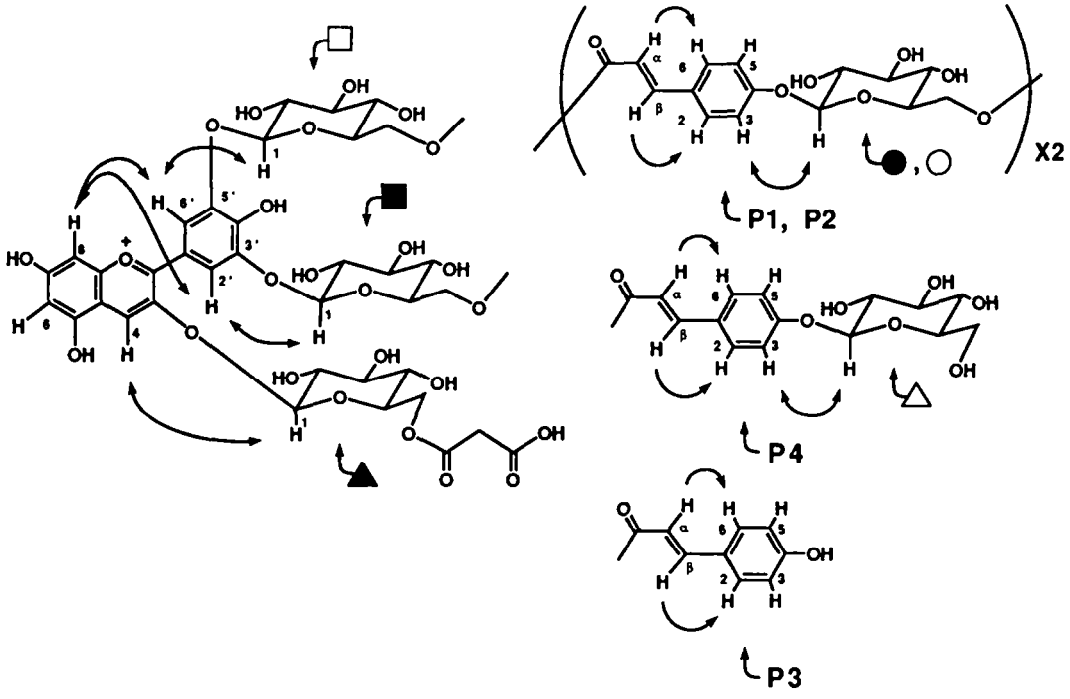


Fig. 1. Partial structures of ternatin B1 with NOE assignments.
The arrows indicate the presence of NOE at 40 °C.

with another two-proton signal (J = 8 Hz; 3 and 5 positions of the p-coumaric acid) in the ^{1}H-^{1}H COSY. These data indicated the presence of four E-p-coumaric acids (P1 - P4). Other five aromatic protons at δ 8.29, 6.61, 6.67, 7.98, and 7.93 were assigned to 4, 6, 8, 2', and 6' protons of a delphinidin nucleus by the spin decoupling ($J_{4,8}$ > 0 Hz, $J_{6,8}$ = 2.0 Hz)[5] and the difference NOE (H-8 ⇄ H-2' and 6'; Fig. 1). Two signals at δ 7.98 and 7.93 corresponding to 2' and 6' positions of the delphinidin nucleus indicated that two substituents on the 3' and 5' positions are not equal.

Table 1. ^{1}H and ^{13}C NMR spectra of ternatin B1 (δ in ppm and J in Hz)

^{1}H NMR (500 MHz, 10% CF_3COOD-CD_3OD, 40°C)

position	□- Glc			■-Glc			○-Glc			●-Glc			△-Glc		
	δ		J	δ		J	δ		J	δ		J	δ		J
1	5.25	d	8	5.29	d	8	4.87	d	8	4.88	d	8	5.00	d	8
2	3.72	t	9	3.76	dd	8,9	3.60	t	9	3.57	t	9	3.53	t	9
3	3.68	t	9	3.69	t	9	3.55	t	9	3.55	t	9	3.56	t	9
4	3.43	t	9	3.47	t	9	3.44	t	9	3.43	t	9	3.47	t	9
5	3.87	t	9	3.92	t	9	3.76	t	9	3.72	t	9	3.52	br.	
6a	4.80	d	12				4.64	d	12	4.64	d	12	3.91	d	12
6b	4.22	dd	9,12	4.33	dd	9,12	4.27	dd	9,12	4.26	dd	9,12	3.75	dd	5,12

position	▲-Glc		
	δ		J
1	4.68	d	8
2	3.77	dd	8,9
3	3.67	t	9
4	3.46	t	9
5	3.78	dd	8,9
6a	4.56	d	12
6b	4.29	dd	8,12

position	anthocyanidin		
	δ		J
4	8.29	br.s	
6	6.61*	d	2
8	6.67*	dd	2
2'	7.98	br.s	
6'	7.93	br.s	

position	P1	P2	P3	P4	
	δ	δ	δ	δ	J
α	5.72	5.84	6.15	6.20	d 16
β	7.01	7.09	7.39	7.39	d 16
2 & 6	6.79	6.86	7.14	7.25	d 9
3 & 5	6.76	6.81	6.67	6.96	d 9

^{13}C NMR (125 MHz, 10% CF_3COOD-CD_3OD, 50°C)

position	P1	P2	P3	P4
	δ	δ	δ	δ
1	130.89	130.89	131.42	131.09
2 & 6	129.63	129.63	127.31	129.78
3 & 5	118.79	118.79		118.65
4	160.03	160.54	161.66	161.24
α	117.55	117.55	115.37	117.36
β	145.35	145.53	147.88	147.31
carbonyl	168.56	168.52	169.30	168.90

position	Glc
	δ
□-1	102.29
■-1	102.54
○-1	101.96
●-1	101.96
△-1	102.22
▲-1	104.45

position	anthocyanidin
	δ
3	146.11
4	135.96
3'& 5'	147.88

position	malonate
	δ
ester carbonyl	169.23

* This proton was exchanged with deuterium within a few months.

Six doublets (J = 8 Hz) at δ 5.25 (□-1), 5.29 (■-1), 4.87 (○-1), 4.88 (●-1), 5.00 (△-1) and 4.68 (▲-1) indicated the presence of six anomeric protons of sugars. Each of the signals showed NOE[6] (Fig. 2) on a two-proton signal of the 3 and 5 positions of one of the p-coumaric acids (P1 - P4) or a signal of one of the protons (H-4, 2' and 6') on the delphinidin nucleus as depicted in Fig. 1. Thus, the position of attachment of the six sugar moieties were established to be as follows: ▲ at 3, □ at 5', and ■ at 3' of the delphinidin nucleus, and ○ at 4 position of P1, ● at 4 of P2, and △ at 4 of P4. That the six sugars are all β-glucopyranosides was deduced from the following coupling constants of the sugar moieties: $J_{1,2} \simeq 8$ Hz and $J_{2,3} \simeq J_{3,4} \simeq J_{4,5} \simeq 9$ Hz, which were obtained by means of HOHAHA (homonuclear Hartmann-Hahn spectroscopy)[7] (Fig. 3). The

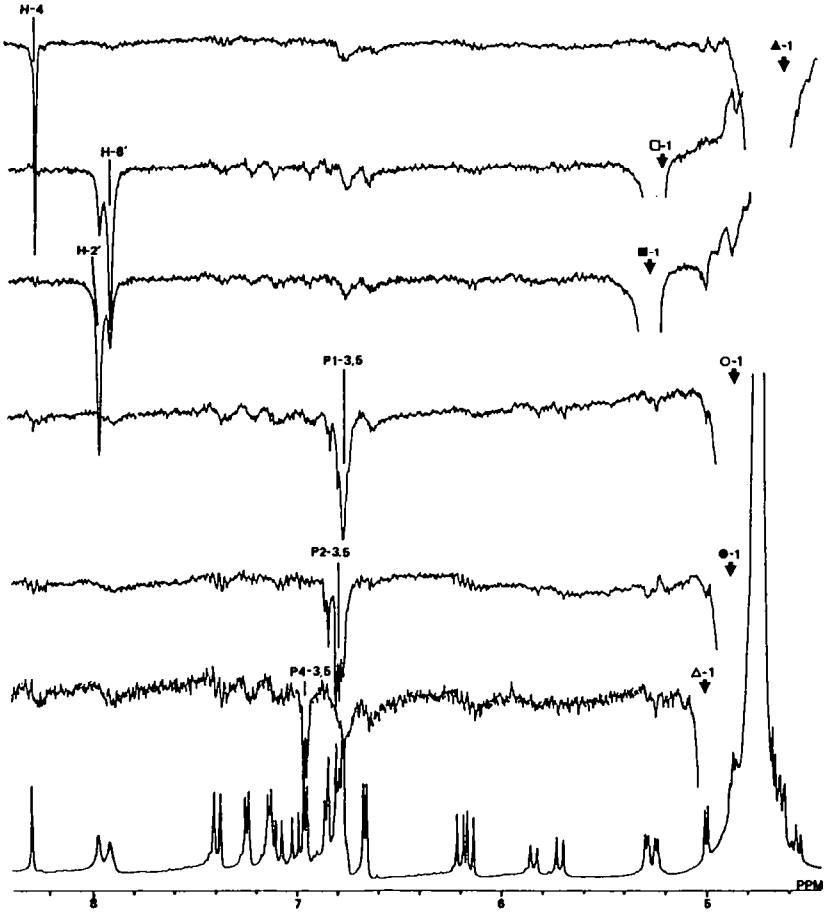


Fig. 2. Negative difference NOE of ternatin B1 between anomeric and aromatic protons. The arrows indicate the position of irradiation.

signals of 6 positions of five glucose moieties (□, ■, ○, ● and ▲) were shifted to lower fields (> 0.5 ppm) than those of 6 position of △-glucose (3.91 and 3.75 ppm), indicating that the five glucose moieties (other than △) are acylated at their 6 position with four p-coumaric acids (P1 - P4) and possibly a malonic acid.

Presence of a malonylglucose unit on 3-position of the delphinidin nucleus was deduced by H_2O_2 oxidation[8] of the pigment and negative FABMS analysis of the product. After oxidation the product was partitioned between ether and water and the water layer was dried up. The negative FABMS analysis of the residue gave a base peak at m/z 266 (M) corresponding to a malonylglucose. Thus, the partial structure of ternatin B1 is deduced as shown in Fig. 1.

The bonds that remained undetermined were the four ester bonds between the p-coumaric acids (P1 - P4) and the glucoses (□, ■, ○, and ●). These were able to be deduced by careful HMBC (heteronuclear multiple bond connection spectroscopy) mesurements[9] (Fig. 4) as indicated with thick bonds in Fig. 5 by observing the correlation between protons at 6 position of a glucose moiety and a proton on a position of a p-coumaric acid through the carbonyl carbon-13 between them. Location of the five glucoside bonds and a malonyl ester bond were also confirmed by the HMBC (Fig. 4) as indicated in Fig. 5. Thus, the complete structure of ternatin B1 was determined as 1 unambiguously.

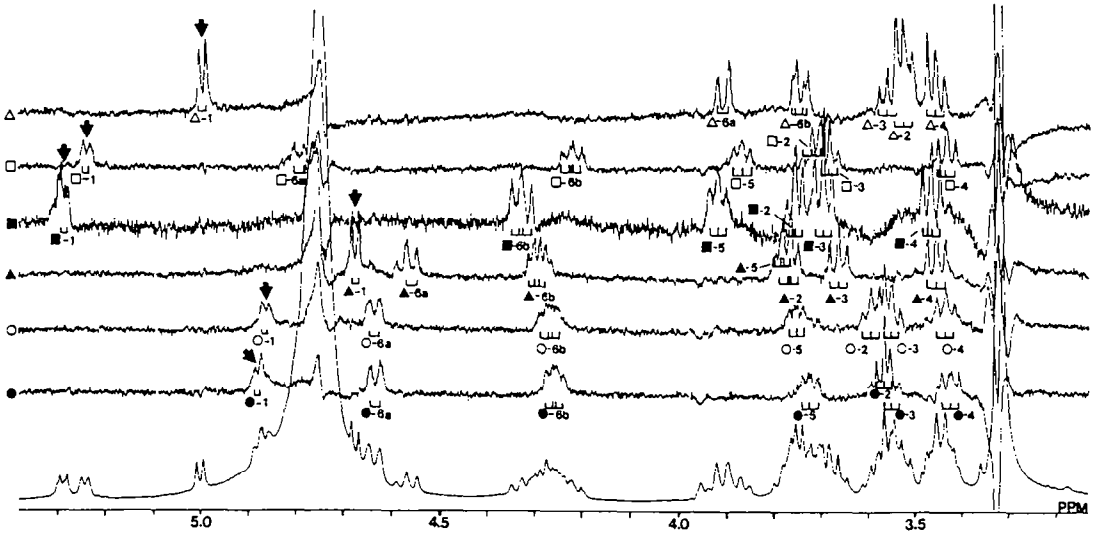


Fig. 3. HOHAHA spectra of ternatin B1 around sugar region, which was measured at 40 °C by irradiation at the anomeric position indicated by an arrow.

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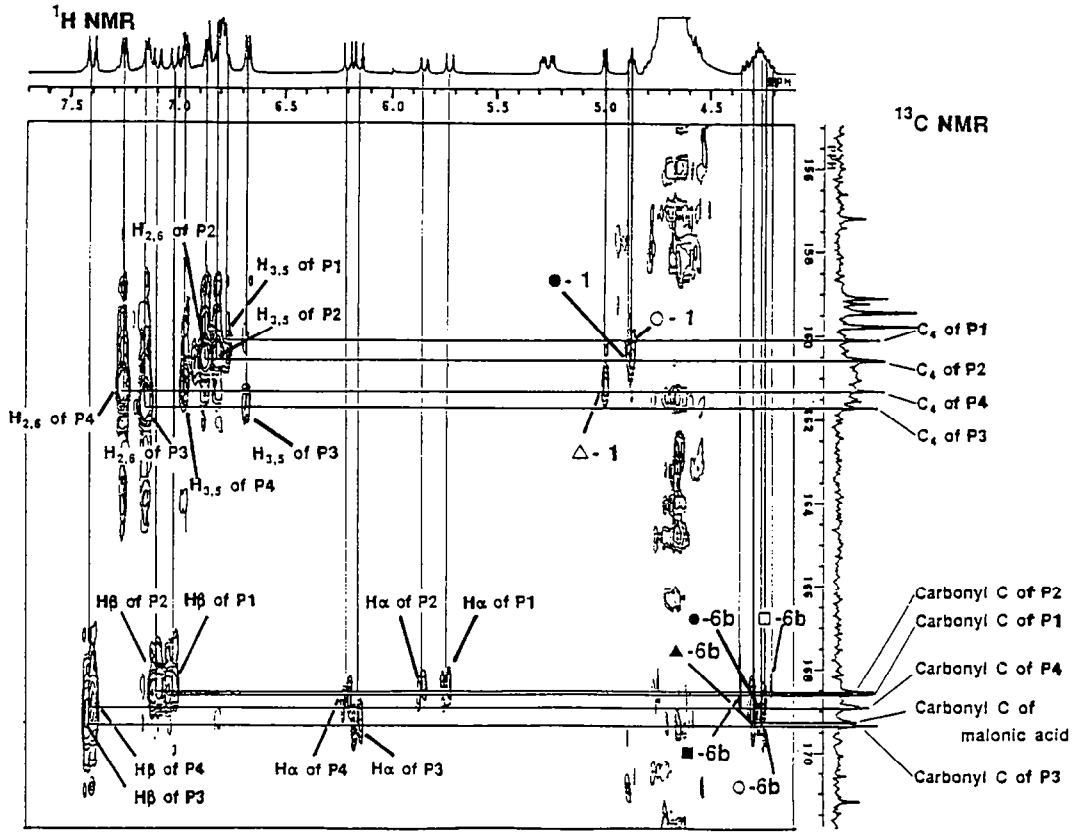

Fig. 4. HMBC spectrum of ternatin B1 measured at 50 °C.

EXPERIMENTAL

General procedures

Electronic spectra were recorded on a Hitachi UV-228 spectrometer. ^{1}H and ^{13}C NMR spectra were obtained on a JEOL GX500 spectrometer operating at 500 MHz for proton and 125 MHz for carbon at variable temperatures using 5-10% TFA*d*-CD_3OD as a solvent. 1D HOHAHA and HMBC spectra were obtained using a pulse sequence supplied from JEOL. The mixing time was set at 60 ms for HMBC. Chemical shifts were recorded as parts per million (ppm) downfield from tetramethylsilane (TMS) as an internal standard.

Positive and negative FABMS spectra were recorded on a JEOL HX-110 and DX7051 spectrometer, respectively, using 1M HCl-*m*-nitrobenzyl alcohol (NBA)-CH_3OH as a matrix. HPLC was carried out using a JASCO 880-PU pump system equipped with a Multi-340 photodiode array detector. Analytical HPLC was carried out using an ODS column (Develosil ODS-5, Nomura Chemicals), which was eluted with a 40% A solution [diluted with H_2O; A = AcOH:MeCN:H_2O (4:5:11)] containing 3% H_3PO_4 and a polymer-supported reverse phase column (Asahipak ODP-50, Asahi Kasei Kogyo) with linear gradient elution from 10% to 30% aq MeCN containing 0.5% TFA in 30 min. For preparative HPLC, an ODS glass column (24 mm ϕ x 250 mm, Develosil LOP-ODS, Nomura Chemicals) and an ODS column (20 mm ϕ x 250 mm, Develosil ODS 10-20, Nomura Chemicals) were used with variously diluted A solution containing 3% H_3PO_4 as an eluant.

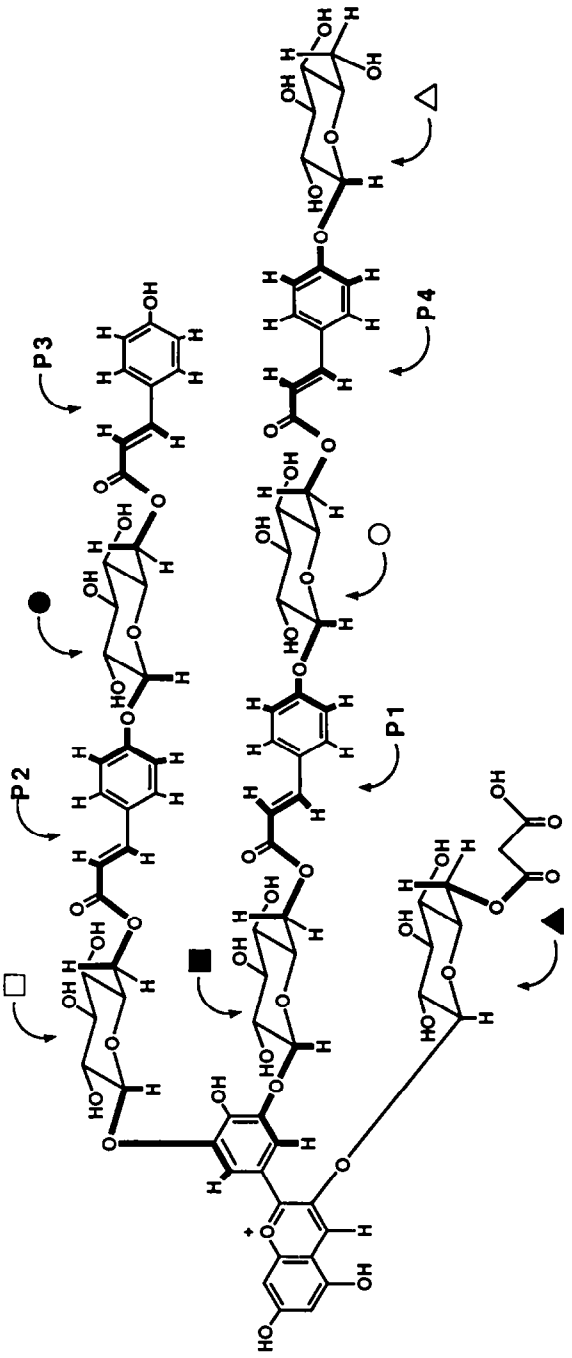

Fig. 5. Structure of ternatin B1 (1)
Thick bonds indicate the bonds deduced by HMBC method.

Isolation of ternatin B1, D1, and D2.

Fresh blue petals of Clitoria ternatea (5.5 Kg) stored at -20 °C were pressed using a piece of cotton cloth at room temp to give blue juice (4 L). To 1.5 L of the juice was added TFA (22.5 ml) under a vigorous stirring. The solution became red. It was centrifuged (2500 rpm x 5 min) and the supernatant filtered through thick filter paper. The filtrate was charged on an Amberlite XAD-7 column (5 cm ϕ x 55 cm). The column was washed with aq. TFA (1.5%, ca 5 L) and eluted stepwise with 10% (1.5 L), 20% (4 L), 25% (2 L), 30% (2 L), and 50% aq. MeCN (1 L) containing 0.5% TFA. Each fraction containing pigments was evaporated below 50 °C. The residue obtained from 30% MeCN fraction containing pigments was diluted with 3% aq H_3PO_4 (400 ml) and adsorbed on a preparative ODS glass column, which was eluted stepwise with 35% (300 ml), 40% (500 ml), 45% (500 ml) and 50% (500 ml) aq A solutions containing 3% H_3PO_4. The fractions containing pigments were diluted with 0.5% aq TFA and adsorbed on the ODS column, which was washed with 0.5% TFA thoroughly to replace the counter anion from phosphate to trifluoroacetate and then eluted with A solution containing 0.5% TFA. The 45% A fraction gave crude pigment (888 mg), which was further purified by ODS HPLC to give ternatin B1 (138 mg) and D2 (77 mg) as amorphous dark red TFA salt. From the 50% A fraction was obtained ternatin D1 (141 mg).
Ternatin B1 TFA salt: UV (2.3×10^{-5} M in 0.01% HCl-MeOH) λ_{max} nm (ε) 550 (25,900), 289 (72,500); positive FABMS (1M HCl-NBA); m/z 1968, 1946, 1697; NMR: see Table 1.
Ternatin D1 TFA salt:[3] UV (2.1×10^{-5} M in 0.01 HCl-MeOH) λ_{max} nm (ε) 550 (23,100), 292 (62,800); positive FABMS (1M HCl-NBA) m/z 1805, 1783 (M), 1534.[4]
Ternatin D2 TFA salt:[2] UV (2.7×10^{-5} M in 0.01% HCl-MeOH) λ_{max} nm (ε) 549 (23,500), 295 (52,400); positive FABMS (1M HCl-NBA) m/z 1474 (M).

H_2O_2 degradation of ternating B1 to malonylglucose.

The degradation reaction was carried out according to Takeda's procedure.[8] To a solution of the ternatin B1 (5 mg) in water (0.5 ml) and MeCN (0.2 ml) was added dropwise 30% aq. H_2O_2 (0.6 ml) for 30 min with stirring under ice-cooling. After 4 hrs 10% Pd on charcoal (ca 10 mg) was added to the colorless solution and the mixture was allowed to stand for 3 hrs. The catalyst was removed by filtration and the filtrate was evaporated to dryness. The residue was treated with 28% aq. ammonia (4 ml) and ethanol (9 ml) at room temp for 14 hrs. The mixture was dried up and partitioned between water and ether (1 ml each). The aqueous layer was dried up to give a yellow solid (4 mg), of which negative FABMS using NBA as the matrix gave peaks at m/z 266 (M), 289 (M + Na), 305 (M + K), and 419 (M + NBA).

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