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New agents for cutaneous photoprotection: derivatives of α-amino acids, 4-aminobenzoic and 4-methoxycinnamic acids

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Summary — Derivatives of sulfur-containing or sulfur-free α-amino acids, 4-aminobenzoic and 4-methoxycinnamic acids, which are potential sun screens, have been synthesized. The effects on melanin formation of 2 compounds (**2** and **12**) were studied *via* enzymatic reactions and cell culture. It was shown that these 2 compounds can enhance pigmentation.

cutaneous photoprotection / melanin formation / 4-aminobenzoic acid / 4-methoxycinnamic acid / sulfur-containing α-amino acids

Introduction

At present, a wide range of sun-blocking preparations have been developed for skin protection. These preparations contain active ingredients that are compounds capable of selectively absorbing harmful solar radiation, *ie* sun screens [1, 2]. Some of these sun screens are reported to pass rapidly through the skin and into the bloodstream [3]. They thus lose their activity and may be harmful to individuals in the case of prolonged use. In order to resolve this problem and improve substantivity, we combined the derivatives of 4-methoxycinnamic and 4-aminobenzoic acids with amino acids by amide function. These 2 groups of acid derivatives are the most widely used as sun screens; amino acids, especially those containing sulfur groups, can bind to the skin surface [4]. It has been suggested that effective levels of cutaneous protection might be best achieved through agents that can enhance pigmentation [5]. In this study, 2 series of new sun screens were synthesized and their inductive activity in melanin synthesis evaluated.

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Abbreviations: PABA: 4-aminobenzoic acid; DOPA: dopamine; DCC: dicyclohexylcarbodiimide; 5-SCD: 5-*S*-cysteinyldopamine.

Chemistry

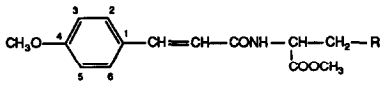
The compounds reported in tables I and II, apart from **10**, **11** and **12**, were synthesized in 2 steps with an overall yield of ≈ 70% (scheme 1). The first step consisted of transforming the 4-methoxycinnamic acid, 4-aminobenzoic acid (PABA) derivatives into the corresponding acid chlorides by treatment with thionyl chloride in chloroform. The final compounds were obtained from the reaction of acid chlorides prepared with the desired amino-acid methyl ester hydrochlorides or their derivatives in the presence of triethylamine; compound **14** was prepared from the reaction of **10** with 4-methoxycinnamic acid chloride in a similar manner. In order to avoid a polymerization reaction from the 2 functional groups of PABA, compounds **10**, **11** and **12** were synthesized through the treatment of PABA with the corresponding amino-acid derivatives in the presence of dicyclohexylcarbodiimide (DCC) [6] (scheme 2).

Results and discussion

Sun-blocking properties

The first criterion for a potential sun screen is its ability to selectively absorb UVB and/or UVA radiation [7]. All the compounds synthesized strongly

Table I. Chemical data and spectral properties of 4-methoxycinnamic acid derivatives.



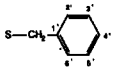
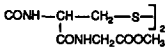
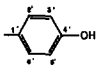
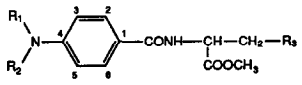
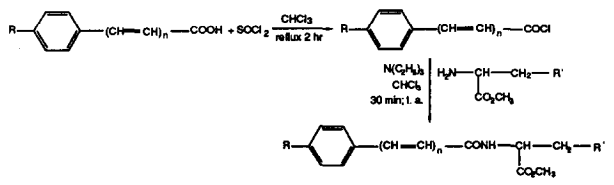
N°	R	mp (C°)	Cryst. solvent $EtOH:H_2O$	Yield (%)	λ_{max} (nm)	ε mol (M^{-1} cm^{-1})
1	CH_2SCH_3	95	1 : 1	78	309	29500
2		170	2 : 1	75	309	46000
3	SH	159	1 : 1	70	314	20000
4	SCH_3	98	1 : 1	78	309	18000
5		128	1 : 1	75	310	24000
6		124	1 : 1	73	309	65000
7		197	1 : 1	70	312	24000
8		188	2 : 1	72	307	24500
9	CH_2COOCH_3	135	1 : 1	76	291	24500

Table II. Chemical data and spectral properties of PABA derivatives.

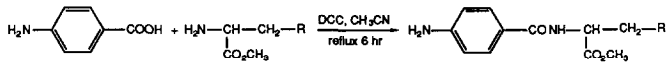


N°	R_1	R_2	R_3	mp (C°)	Cryst. solvent	Yield (%)	λ_{max} (nm)	ε mol (M^{-1} cm^{-1})
10	H	H	CH_2SCH_3	130	Acetone: IPE* 4 : 1	50	280	39200
11	H	H	SH	140	4 : 1	40	277	35500
12	H	H		132	$EtOH$	45	289	45000
13	H	$COCH_3$		169	$EtOH:H_2O$ 1 : 1	75	270	44250
14	H		CH_2SCH_3	133	1 : 1	70	290	31700
15	CH_3	CH_3	CH_2SCH_3	85	1 : 1	75	307	25500
16	CH_3	CH_3		210	1 : 1	72	290	57000

* IPE: isopropyl ether



Scheme 1. $n = 0$ or 1.



Scheme 2.

absorb UV radiation between 260 and 360 nm with an absorption maximum situated in the UVB and a molar absorptivity > 18 000 M^{-1} cm^{-1} (tables I, II). Compared with the original sun screen, PABA, the new compounds retain the same spectral properties with regard to both absorption maximum and molar absorptivity.

Effect on melanin formation from enzymatic reactions

Melanization refers to the whole biological process that leads to the melanin pigmentation of the skin [8]. The melanin precursor is tyrosine. The transformation of tyrosine into melanin requires enzymatic (hydroxylation of tyrosine to DOPA and oxidation of DOPA to dopaquinone under the effect of tyrosinase) and non-enzymatic oxidation (non-enzymatic oxidation of DOPA or 5-*S*-cysteinyldopa (5-SCD) to different intermediates that polymerize to produce melanin). The effects of the prepared compounds on melanin formation were first studied through enzymatic reactions. The formation of dopachrome from tyrosine or from DOPA in the absence or in the presence of the new compounds was quantified by UV spectrophotometry at 280 and 475 nm. In order to compare the results, the effect of PABA on melanin formation was studied at the same time. PABA was selected as a control compound as it was already present in numerous anti-solar preparations [9].

Figs 1A and 1B show the effects of PABA, compounds **2** and **12** on dopachrome formation from tyrosine and DOPA respectively. It can be seen that compound **2** like compound **12** increased dopachrome formation by 20% from both tyrosine and DOPA. However, PABA completely prevented dopachrome formation from tyrosine. Notwithstanding, after a

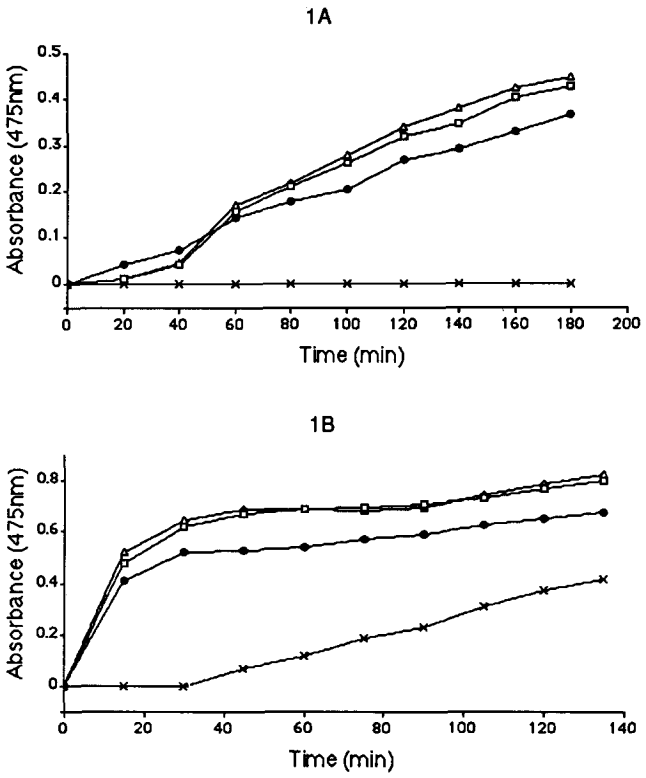


Fig 1. Variation of dopachrome formation from tyrosine (1A) and DOPA (1 B) with time, under action of tyrosinase, in the absence (●) or presence of PABA (✖), compounds **2** (△) or **12** (□) at 2 mM.

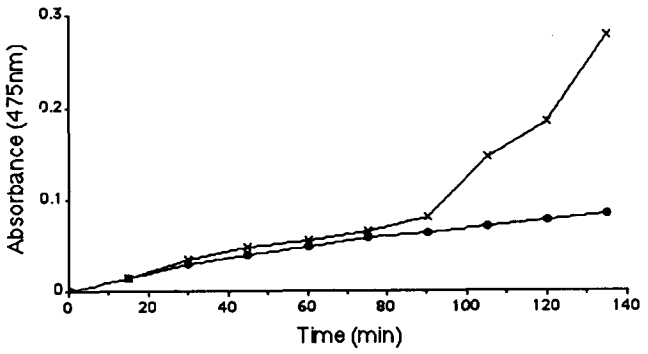


Fig 2. Variation of dopachrome formation from non-enzymatic oxidation of DOPA with time in the absence (●) or presence of PABA (✖) at 2 mM.

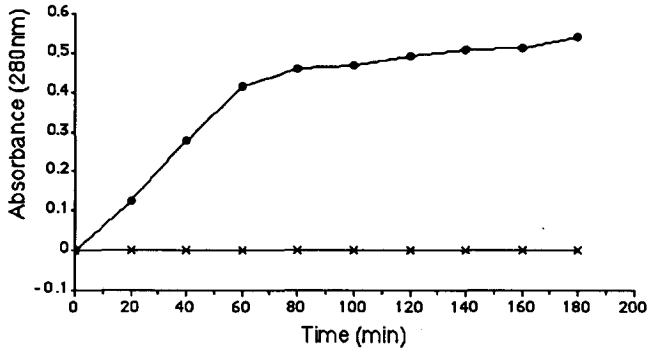


Fig 3. Variation of DOPA formation from tyrosine with time, under action of tyrosinase, in the absence (●) or presence of PABA (✖) at 2 mM.

period of latency, PABA increased the rate of dopachrome formation from DOPA. Since both enzymatic and non-enzymatic DOPA oxidations can take place in forming dopachrome, the effect of PABA on DOPA oxidation in the absence of tyrosinase was assessed. The result obtained shown in figure 2 indicated that PABA can increase the non-enzymatic oxidation of DOPA. Figure 3 demonstrates the effect of PABA on hydroxylation of tyrosine to DOPA under the effect of tyrosinase. The fact that no dopachrome was formed in this case indicates that PABA entirely inhibited the tyrosinase hydroxylase activity. By comparing the chemical structures and the effects on melanin formation of PABA and compound **12**, it is suggested that the acid function of PABA is responsible for the observed inhibitory effect. In fact, it is known that benzoic acid inhibits tyrosinase by acting as a substrate [10].

Effect on melanin formation in the cell culture

The effect of the new compounds on melanin formation was then evaluated *in vitro* using cell cultures. The quantities of melanin extracted from melanomic cells, cultured in the absence or presence of the test compounds, were determined by spectrophotometry at 400 nm. Figure 4 presents the percentages of the variations in melanin concentrations induced by PABA, **2** and **12** in comparison with the untreated cell cultures. The concentration used for the 3 compounds was 0.1 mM; below this concentration, a non-significant difference could be observed for the variation in melanin quantities.

The addition of the 3 compounds to the culture medium induced an increase in OD in the order of 30–80% relative to the untreated culture. For compounds **2** and **12** a strong increase in the pigmentation of the cell cultures began on the 4th d, and for PABA on the 5 th d. These results are consistent with those obtained through enzymatic reactions.

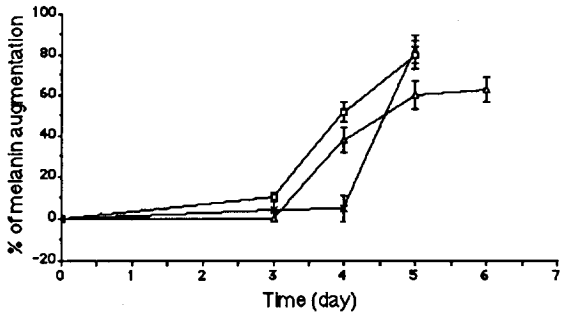


Fig 4. Augmentation of melanin concentration by PABA (✖), compounds **2** (△) and **12** (□) in the cell culture, expressed as % of the control.

Effect on melanin precursor formation

DOPA and 5-SCD are the 2 important melanin precursors [11, 12]. In order to determine at which point a compound causes an increase in melanin synthesis, we measured the quantities of the 2 melanin precursors, DOPA and 5-SCD. Figures 5A and 5B respectively show the percentages of variation in the quantities of DOPA and 5-SCD induced by 0.1 mM PABA, **2** and **12** compared with untreated culture. Compounds **2** and **12** led to an increase in the percentages of variation of both DOPA and 5-SCD. However, PABA showed an opposite effect.

These results were in accordance with those obtained from enzymatic reactions, indicating that compounds **2** and **12** act upon the formation of both precursors and melanin. PABA inhibits tyrosinase hydroxylase activity, and as a result inhibits the formation of melanin precursors. In return, PABA increases the non-enzymatic oxidation of DOPA, bringing about an increase in the melanin formation.

In conclusion, the results of this study illustrate that besides their strong absorption of UVB, the new compounds selected for this study, **2** and **12**, can enhance melanization. This novel property appears to be highly beneficial regarding cutaneous protection against the harmful effects of UV radiation.

Experimental protocols

Chemistry

Melting points were determined on a Kofler hot-stage apparatus. The infrared (IR) spectra of solid samples were run as KBr disks on a Beckman 4230 spectrophotometer. ^{1}H-NMR spectra were recorded on a Brüker AC 200 MHz spectrometer. Chemical shifts are given in ppm (δ) downfield from tetramethylsilane (TMS).

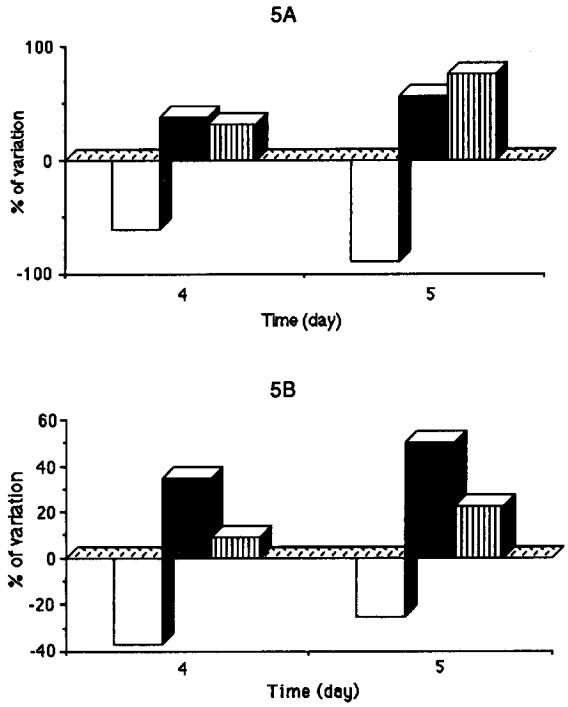


Fig 5. Variation of DOPA (5A) and 5-SCD (5B) concentrations from tyrosine induced by PABA (□), compounds **2** (■) and **12** (▥) in the cell culture, expressed as % of the control.

The synthesis of new compounds **1–9** and **13–16** was carried out using the same synthetic procedures. The protocol for compound **2** has therefore been described as a representative example.

Synthesis of 4-methoxycinnamic acid chloride

To a solution of 4-methoxycinnamic acid (28.5 g, 0.16 mol) in 200 ml chloroform, thionyl chloride (18 ml, 0.25 mol) was added dropwise. The mixture was heated to reflux for 2 h. The solvent was removed *in vacuo*. Chloroform (30 ml) was added to the residue and evaporated *in vacuo*. This process was repeated 3 times in order to completely eliminate the excess thionyl chloride.

Synthesis of N,N'-bis(4-methoxycinnamoyl)-L-cystine dimethyl ester **2**

A solution of 4-methoxycinnamic acid chloride (23.6 g, 0.12 mol) previously prepared in 60 ml chloroform was slowly added to a solution of L-cystine dimethyl ester dihydrochloride (30.7 g, 0.09 mol) and 60 ml (0.43 mol) triethylamine in 150 ml chloroform (in an ice bath). After stirring for 30 min at room temperature, the solution was washed several times with 1 N aqueous HCl solution, then with 10% aqueous $KHCO_3$ solution, dried over Na_2SO_4 and evaporated. The residual oil was precipitated in cyclohexane. The product was crystallized from a mixture of EtOH/H_2O (2:1) to afford 70.7 g (75%) white crystals, mp: 169–171°C. IR (KBr): 3300 (NH amide), 1730 (C=O ester), 1650 (C=O amide), 1635 (C=C ethylenic), 1600 cm^{-1} (C=C arom). ^{1}H-NMR (DMSO–d_6) δ: 3.20 (dd, 4H, CH_2-S), 3.72 (s, 6H, OCH_3), 3.79 (s, 6H, $COOCH_3$), 5.08 (m,

2H, CH), 6.25 (d, 2H, CH=, J = 16 Hz), 6.82 (d, 4H, $H_3 + H_5$), 6.89 (d, 2H, NH), 7.45 (d, 4H, $H_2 + H_6$), 7.65 (d, 2H, =CH, J = 16 Hz). Physical data for compounds **1**, **3–9**, **13–16** have been summarized in table III.

Compounds **10–12** were synthesized by a similar synthetic procedure. The protocol described below for compound **10** has been given as an example.

*Synthesis of N-(4-aminobenzoyl)-L-methionine methyl ester **10***

A mixture of L-methionine methyl ester (2 g, 12.3 mmol) and dicyclohexylcarbodiimide (DCC) (3.0 g, 14.5 mmol) in 40 ml acetonitrile was heated to reflux for 6 h. The solution was cooled and filtered and the solvent evaporated. Chloroform (30 ml) was added to the residue and the organic phase extracted in 2 portions (30 ml) of 1 N aqueous HCl solution. The combined aqueous phases were neutralized with a 15% aqueous NH_4OH solution, then extracted 3 times with chloroform (30 ml). The combined organic phases were dried over Na_2SO_4, evaporated under reduced pressure, and the resulting solid crystallized from a mixture of acetone/isopropyl ether (4:1) to afford 1.70 g (50%) white crystals, mp: 129–131°C. IR (KBr): 3440 (NH amide), 3230–3350 (double bands, NH_2), 1735 (C=O ester), 1625 (C=O amide), 1600 cm^{-1} (C=C arom). ^{1}H-NMR ($CDCl_3$) δ: 2.12 (s, 3H, SCH_3), 2.20 (m, 2H, -CH-CH_2), 2.60 (t, 2H, CH_2S), 3.79 (s, 3H, OCH_3), 4.00 (s, 2H, NH_2), 4.90 (m, 1H, -$CHCH_2$), 6.67 (d, 2H, $H_3 + H_5$), 6.75 (d, 1H, NH), 7.65 (d, 2H, $H_2 + H_6$). Physical data for compounds **11** and **12** have been summarized in table III.

Biochemistry

Assay of melanin produced by enzymatic and non-enzymatic reactions

Tyrosine, DOPA and 5-SCD used in this study were obtained from Fluka Chemical Co (France); tyrosinase was from Sigma Chemical Co (France). In a quartz cell, 1 ml phosphate buffer solution (0.142 M Na_2HPO_4 and 0.029 M citric acid) (pH 6.5) and the test compound were added to a mixture of tyrosine (or DOPA) and tyrosinase in the same buffer. The final concentrations of the test compound, tyrosine, DOPA and tyrosinase were respectively 2.0 mM, 1.6 mM, 2.0 mM and 100 µg/ml. The OD was measured every 20 min at 280 nm for DOPA formation and at 475 nm for dopachrome formation.

The formation of DOPA from tyrosine and dopachrome from DOPA in the absence and in the presence of PABA was quantified in the same manner as described above. The experimental conditions used remain the same only the addition of tyrosinase was omitted.

Assay of melanin produced by cell cultures

The IGR 37 cell line used was produced at the Gustave Roussy Institute of Villejuif from a human metastatic tumor. It was cultured in 175 cm^2 culture flasks in minimum essential medium (MEM) supplemented with 9 µM proline, 25 µM vitamin C and 5% fetal calf serum. The medium was renewed twice weekly and the cultures replated once weekly. The cultures were maintained at 37°C in a humid atmosphere with 5% CO_2 content.

On the first day, 1 x 10^6 cells were introduced into culture flasks containing 50 ml of the previously described medium. On the second day, the first medium was replaced by a new medium containing 0.1 mM test compound. Prior to its incorporation into the medium, the test compound was dissolved in DMSO. Each DMSO solution in the medium was present at a maximum concentration of 0.1%. The medium obtained in this manner underwent sterile filtration using 0.22-µm filters. On

Table III. Physical data for compounds **1**, **3–9**, **11–16**.

Compounds	IR (KBr) ν (cm^{-1})	^{1}H NMR (DMSO-D_6), δ (ppm)
1	3300 (NH amide) 1740 (C=O ester) 1650 (C=O amide) 1635 (C=C ethylenic) 1600 (C=C arom)	2.10 (s, 3H, S-CH_3), 2.12 (t, 2H, CH_2-S), 2.50 (q, 2H, $CHCH_2$), 3.72 (s, 3H, OCH_3), 3.82 (s, 3H, $COOCH_3$), 5.00 (q, 1H, CH-CH_2), 6.25 (d, 1H, CH=, J = 16 Hz), 6.80 (d, 1H, NH), 6.82 (d, 2H, H_3+H_5), 7.45 (d, 2H, H_2+H_6), 7.65 (d, 1H, =CH, J = 16 Hz).
3	3300 (NH amide) 1730 (C=O ester) 1670 (C=O amide) 1635 (C=C ethylenic) 1600 (C=C arom)	1.35 (t, 1H, SH), 3.20 (dd, 2H, CH_2-S), 3.72 (s, 3H, OCH_3), 3.79 (s, 3H, $COOCH_3$), 6.25 (d, 1H, CH=, J = 16 Hz), 6.82 (d, 2H, H_3+H_5), 6.89 (d, 1H, NH), 7.45 (d, 2H, H_2+H_6), 7.65 (d, 1H, =CH, J = 16 Hz).
4	3300 (NH amide) 1720 (C=O ester) 1650 (C=O amide) 1635 (C=C ethylenic) 1600 (C=C arom)	2.10 (s, 3H, S-CH_3), 3.05 (d, 2H, CH_2), 3.72 (s, 3H, OCH_3), 3.80 (s, 3H, $COOCH_3$), 5.02 (q, 1H, CH), 6.25 (d, 1H, CH=, J = 16 Hz), 6.82 (d, 2H, H_3+H_5), 7.00 (d, 1H, NH), 7.45 (d, 2H, H_2+H_6), 7.65 (d, 1H, =CH, J = 16 Hz).
5	3300 (NH amide) 1740 (C=O ester) 1650 (C=O amide) 1635 (C=C ethylenic) 1600 (C=C arom)	2.90 (t, 2H, CH_2-C_6H_5), 3.72 (s, 3H, OCH_3), 3.82 (s, 3H, $COOCH_3$), 3.90 (t, 2H, CH-CH_2), 5.02 (q, 1H, CH), 6.25 (d, 1H, CH=, J = 16 Hz), 6.80 (d, 1H, NH), 6.82 (d, 2H, H_3+H_5), 7.00 (d, 1H, NH), 7.15 (m, 5H, H_2'+H_3'+H_4'+H_5'+H_6'), 7.45 (d, 2H, H_2+H_6), 7.65 (d, 1H, =CH, J = 16 Hz).
6	3300 (NH amide) 1740 (C=O ester) 1660 (C=O amide) 1635 (C=C ethylenic) 1600 (C=C arom)	2.25 (m, 6H CH_2, CH_2, CH_2), 3.67 (s, 3H, $COOCH_3$), 3.72 (s, 3H, OCH_3), 3.77 's, 3H, $COOCH_3$), 5.05 (m, 2H, CH, CH), 6.25 (d, 1H, CH=, J = 16Hz), 6.75 (m, 3H, NH, NH, NH), 6.82 (d, 2H, H_3+H_5), 7.45 (d, 2H, H_2+H_6), 7.65 (d, =CH, J = 16Hz)
7	3450 (OH) 3300 (NH amide) 1730 (C=O ester) 1650 (C=O amide) 1635 (C=C ethylenic) 1600 (C=C arom)	3.20 (m, 2H, CH_2), 3.72 (s, 3H, OCH_3),3.80 (s, 3H, $COOCH_3$), 5.02 (m, 1H, CH), 5.5 (m, 1H, CH=), J = 16Hz), 6.72 (d, 2H, H_3+H_5), 6.82 (d, 2H, H_3+H_5), 6.90 (d, 1H, NH), 7.05 (d, 2H, H_2+H_6'), 7.45 (d, 2H, H_2+H_6), 7.65 (d, 1H, CH=, J = 16 Hz)
8	3350 (NH amide) 3230 (NH imidazol) 1750 (C=O ester) 1650 (C=O amide) 1635 (C=C ethylenic) 1600 (C=C arom)	2.82 (m, 2H, CH_2), 3.72 (s, 3H, OCH_3), 3.82 (s, 3H, $COOCH_3$), 5.02 (m, 1H, CH), 6.25 (d, 1CH, CH=), 6.82 (d, 2H, H_3+H_5), 7.02 (d, 1H, NH), 7.20 (d, 1H, H_5'), 7.45 (d, 2H, H_2+H_6), 7.65 (d, 1H, =CH), 7.70 (s, 1H, H_2')
9	3350 (NH amide) 1720 (C=O esters) 1650 (C=O amide) 1635 (C=C ethylenic 1600 (C=C arom)	2.20 (m, 4H, CH_2CH_2), 3.72 (s, 3H, OCH_3), 3.75 (s, 3H, $COOCH_3$), 3.80 (s, 3H, $COOCH_3$), 4.80 (m, 1H, CH=, J = 16Hz), 6.82 (d, 2H, H_3+H_5), 7.05 (d, 1H, NH), 7.45 (d, 2H, H_2+H_6), 7.65 (d, 1H, =CH, J = 16Hz)
11	3440 (NH amide) 3230-3350 (double bands NH_2) 1735 (C=O ester) 1625 (C=O amide) 1600 (C=C arom)	3.17 (m, 2H, CH_2S), 3.40 (m, 2H, NH_2), 3.64 (s, 3H, OCH_3), 4.69 (m, 1H, CH), 6.55 (d, 2H, H_3+H_5), 7.58 (d, 2H, H_2+H_6), 8.40 (d, 1H, NH)
12	3440 (NH amide) 3230-3350 (double bands NH_2) 1735 (C=O ester) 1630 (C=O amide) 1600 (C=C arom)	3.20 (m, 2H, CH_2S), 3.64 (s, 3H, OCH_3), 4.68 (m, 1H, CH), 5.71 (m, 2H, NH_2), 6.57(d, 2H, H_3+H_5), 7.55 (d, 2H, H_2+H_6), 8.40 (d, 1H, NH)
13	3300 (NH amide) 1740 (C=O ester) 1670 (C=O amide arom) 1630 (C=O amide) 1600 (C=C arom)	2.10 (s, 3H, CH_3CO), 2.25 (m, 2H, CH_2S), 2.50 (m, 1H, NH, NH), 3.78 (s, 3H, OCH_3), 4.92 (m, 1H, CH), 7.60 (d, 2H, H_3+H_5), 7.85 (d, 2H, H_2+H_6)
14	3300 (NH amides) 1740 (C=O ester) 1670 (C=O amide) 1650 (C=O amide) 1600 (C=C arom)	2.15 (s, 3H, SCH_3), 2.25 (m, 2H, -$CHCH_2$), 2.68 (t, 2H, CH_2S, 3.72 (s, 3H, OCH_3), 3776 (s, 3H, $COOCH_3$), 5.06 (m, 1H, CH), 6.20 (d, 1H, CH=), 6.64 (d, 2H, H_3+H_5'), 6.99 (d, 1H, NH-CH), 7.20 (d, 2H, H_2+H_6'), 7.65 (d, 1H, =CH), 7.43 (d, 2H, H_2+H_6), 7.72 (d, 2H, H_3+H_5), 8. 20 (s, 1H, NH)
15	3300 (NH amide) 1740 (C=O ester) 1630 (C=O amide) 1600 (C=C arom)	2.13 (s, 3H, SCH_3), 2.28 (m, 2H, -$CHCH_2$), 2.58 (m, 2H, CH_2S), 3.01 (s, 6H, $N(CH_3)_2$), 3.78 (s, 3H, OCH_3), 4.92 (m, 1H, CH), 6.68 (d, 2H, H_3+H_5), 7.71 (d, 2H, H_2+H_6)
16	3360 (NH amide) 1740 (C=O ester) 1630 (C=O amide) 1600 (C=C arom)	3.05 (s, 6H, $N(CH_3)_2$), 3.32 (m, 2H, CH_2), 3.76 (s, 3H, OCH_3), 5.06 (m, 1H, CH), 6.64 (d, 2H, H_3+H_5), 6.99 (d, 1H, NH), 7.72 (d, 2H, H_2+H_6)

the 4th d, the cells were separated with a trypsin solution and counted with a haemocytometer. The cells were then centrifuged at 3000 rpm for 10 min; the supernatant was then removed, leaving the cell pellets which were stored at –20°C.

Each experiment was carried out using a minimum of 4 flasks. A minimum of 4 control flasks were cultured under the same conditions in the presence of 0.1% DMSO. All operations were effected in the absence of UV radiation; the flasks and in particular the solutions were protected with aluminum foil. The assay was carried out according to the method of Whittaker [14]. The cell pellets were extracted 3 times with 5% trichloroacetic acid at 4°C, twice with ether/ethanol (1:3) at 4°C and once with absolute ether at room temperature. The dry extracted residue was dissolved in 1.0 ml 0.85 N KOH, heated to 100°C for 10 min, then cooled to room temperature. OD measurements were carried out at 400 nm [15] with a Beckman DU spectrophotometer. Melanin content was expressed in terms of OD/cell.

Assay of melanin precursors produced by cell cultures

The melanomic cells were cultured in the same manner as in the previous study. The cell pellets were then dissolved in 4 N perchloric acid and centrifuged at 48 000 *g* for 15 min at 4°C. The supernatants were directly recovered into centrifuge tubes containing a pH 4 mixture of alumina and sodium EDTA. Then each tube was adjusted to pH 8.6 (± 0.01) with 5 N KOH. After 15 min agitation, the tubes were centrifuged at 5600 rpm for 5 min at 4°C. The supernatant was discarded and the alumina–sodium EDTA mixture washed with pH 8.6 phosphate buffer. The operation was repeated twice. After the second centrifugation, the alumina–sodium EDTA mixture was added to 0.5 N perchloric acid and agitated for 15 min, the tubes were then centrifuged at 4600 rpm for 5 min at 4°C. The supernatant was removed and filtered. The Melanin precursor assay was carried out immediately *via* HPLC, or the supernatant was frozen in a homolysis tube for later assay. The conditions were as follows: ODS Ultrasphere column (3 μm, 4.6 mm x 7.5 cm), solvent system: methanol 3 mM/orthophosphoric acid (5:95) at a flow rate of 1.5 ml/min. The respective retention times of DOPA and 5-SCD under these conditions were 2 and 4.5 min.

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