Eur. J. Med. Chem. 35 (2000) 797−803

S0223523400001811/FLA

Original article

Synthesis of ionones and carvone analogues: olfactory properties and preliminary toxicity assays

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Received 28 December 1999; revised 21 February 2000; accepted 24 February 2000

Abstract – Vilsmeier reagents react with α/β-ionones and carvone to produce aldehydes **7**–**11** in a one-step procedure. The indene derivative **11**, which came from the double iminoalkylation of carvone and ring closure with the elimination of dimethylamine, was practically odourless, while all the others had peculiar odours which were very different from the starting material. The cytotoxicity data of **9** and **10**, which are the most promising potential perfume ingredients, are also reported. © 2000 Éditions scientifiques et médicales Elsevier SAS

ionone analogues / carvone analogues / odoriferous substances / aldehydes / cytotoxicity / Vilsmeier reaction

1. Introduction

Aldehydes and ketones have played a primary role in perfumery and continue to be one of the leading choices in perfume composition [1]. While ketones are widely represented in nature, the majority of aldehyde derivatives come from synthesis. Moreover, among the monocyclic terpenes, aldehydes occur in very low concentrations in essential oils and are seldom used. On the other hand, ketones having the p-menthane or (trimethylcyclohexenyl)alkenone skeletons **A** and **B** are commercially relevant as fragrance and flavour compounds (*figure 1*).

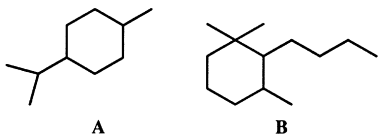

Figure 1. p-Menthane and (trimethylcyclohexenyl)alkenone skeletons **A** and **B**.

Various substances resulting from a combination of conjugate or non-conjugate double bonds with the carbonyl group in correlation with the natural pattern of the 2,6,6-trimethyl or 1-methyl-4-isopropyl substituents have been isolated or have been prepared and studied for their olfactory properties. Among them are comprised famous fragrances of high impact such as menthones, carvones, ionones and damascones.

A very large number of cyclohexane and cyclohexenecarboxaldehydes have been synthesised. Some of them showed outstanding odoriferous properties like the simple 2,4-dimethyl-3-cyclohexene carboxaldehyde **1** (Cyclal-Givaudan) [2] or the more complex 4-(4-methyl-3-penten-1-yl)-3-cyclohexene carboxaldehyde **2** (Myrac aldehyde-IFF) [2, 3] and its hydroxy derivative **3** (Lyral-IFF) [3] (*figure 2*).

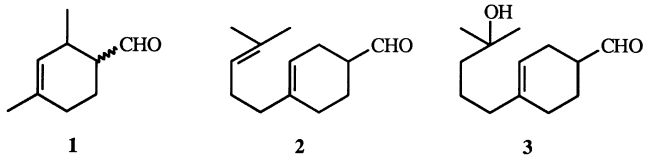

Figure 2. Compounds **1**–**3**.

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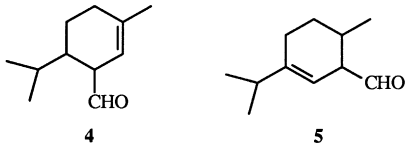

Figure 3. Aldehydes **4** and **5**.

Only a few can be correlated to p-menthane **A** and generally do not possess noticeable olfactory properties. To the best of our knowledge only aldehydes **4** and **5** are reported to be used as perfuming agents [4] (*figure 3*).

Moreover, among the numerous aldehydes which can be correlated to the structure **B**, only Ambra aldehyde **6**, which comes from the degradation of Ambrein, has been studied and synthesised for its outstanding marine-ozone odor [5] (*figure 4*).

To obtain more insight regarding how the introduction of an aldehyde into structures **A** and **B** influences the odour, we planned to formylate the commercially available α- and β-ionones and carvone.

2. Chemistry

Vilsmeier reagents have been applied in numerous synthetic transformations of acyclic and cyclic carbonyl compounds to give several distinct types of products whose nature prevalently depends on the substituents. Among them mono- and polyformylated products are the usual outcome [6]. When α- and β-ionones were reacted with the Vilsmeier reagent N,N-dimethyl-(diethyl)formamide/phosphorus oxychloride (VR), the expected β-chlorovinylaldehydes **7** were obtained together with the unprecedented enaminones **8** [7] (*figure 5*). The latter, on the other hand, besides having an interesting odour, have proven to be useful key intermediates in synthesising new short retinoid-like compounds [8]. We were also able to obtain compounds **7** or **8** separately, thus improving the yields, simply by modifying the workup (see *figure 5* and Experimental protocols).

Transfer of the reaction method from the ionones to the carvone was successful, but some conditions needed to be

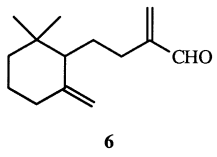

Figure 4. Ambra aldehyde **6**.

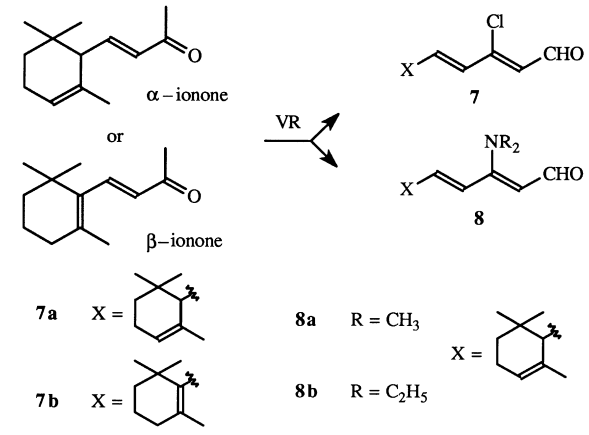

Figure 5. Synthesis of compounds **7** and **8**.

changed carefully in order to improve separately the yield of each compound obtained. In fact, the Vilsmeier formylation of carvone afforded the mixture of the expected formylcyclohexadiene **9** together with the 2-chlorobenzaldehyde **10** and the indene derivative **11** (*figure 6*).

Modifying the reaction time resulted in improved yields of each compound. Any attempt to further increase the yields, such as changing the temperature and the molar rate from the typical procedure, failed. We speculate that this may be due to the formation of other (poly)formylated products which come from the more active position of intermediate **12** and **13**. These by-products end up in an unworkable thick brownish mixture (see *figure 7* and Experimental protocols).

The reaction pathways depicted in *figure 7* account for the formation of the indene derivative **11** and can give an explanation to the critical reaction. Carvone undergoes iminoalkylation to give cation **12**, which is the source of **9** after hydrolysis. Deprotonation of **12** affords **13**, which may be further iminoalkylated to give species **14**, which affords **15** by ring closure with elimination of dimethylamine. Finally, benzaldehyde **10** comes from the oxidative aromatization of **9**.

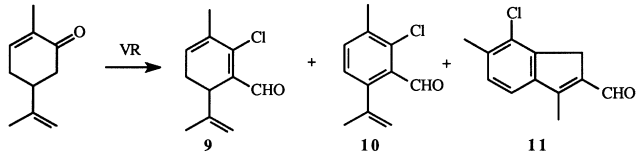

Figure 6. Synthesis of compounds **9**–**11**.

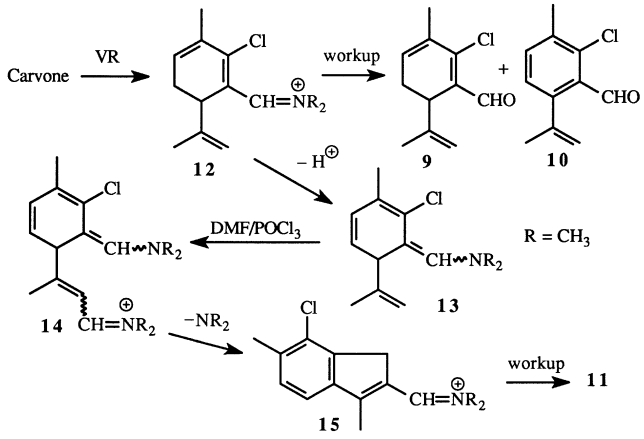

Figure 7. Synthesis of compound **11**.

The formation of **11** accounts for further iminoalkylation on the unactivated double bond of the isopropenyl group. This second step, which leads to intermediate **14**, has to be highly stereoselective, since only isomer *E* is able to give **15** by ring closure. That the thermodynamic equilibrium was in favour of the *E* isomer has been supported by the yield increase of **11** with reaction time. Analogous equilibration in favour of the *E* form was observed in the Vilsmeier formylation of limonene [9].

All new compounds were characterised by their elemental analyses and by IR, ^{1}H- and ^{13}C-NMR spectra (see Experimental protocols). The structure of **11** was also secured by X-ray crystallographic analysis [10].

3. Toxicology

To evaluate the cytotoxicity of compounds **9** and **10**, the human keratinocyte NCTC 2544 cell line was used. We applied three in vitro tests, which are increasingly used as cost-effective and rapid methods in the prescreening phase in the development of new drugs and other chemicals [11].

One of the most widely used cytotoxicity assays (NRU) is based on the ability of viable cells to incorporate and bind neutral red, which is a vital, weakly cationic, dye; it penetrates cell membranes by non-ionic passive diffusion and concentrates in the lysosomes, where it binds to various groups of the lysosomal matrix by electrostatic hydrophobic bonds [12].

In the MTT assay, the yellow MTT tetrazolium [2-(4,5-dimethyl-2-thiazolyl)3,5-dipheny-2H-tetrazolium bromide] is reduced in viable cells to a purple coloured MTT-formazan precipitate by mitochondrial dehydrogenase enzymes.

Finally, the evaluation of total protein content (TPC) was used as a measure of surviving cells still attached to the monolayer support [13]. Sodium dodecylsulphate (SDS) was used as positive control.

4. Results and discussion

4.1. Olfactory properties

Table I summarises the olfactory properties and potential use of the aldehyde derivatives **7**–**11**.

Natural α-ionone possesses a sweet-floral note reminiscent of violets while β-ionone, reminiscent of cedarwood, changing to violet upon dilution. These descriptors are completely lost in **7a**, revealing instead a bitter, tarry, unpleasant note. In **7b** a sweet-floral note recalling that of β-ionone remains, but is stronger, more 'medicinal' and woody. In sharp contrast, **8a** possesses a weak but persistent fragrance of interesting and pleasant character: an iron-like tonality occurring in orris oil accompanied by a vanillic note and a 'poudrée' subnote, which are the typical olfactory notes of red lipsticks having orris and vanilla components. On the other hand, **8b** has a fruity note, reminescent of cooked prunes and tagetes oil and then evolves into a saffron and ionone-like note.

The formylcyclohexadiene **9** exhibits an extremely strong but pleasant bitter note with a woody fragrance, evolving towards a typical quinoline note. The characteristics of the odour change completely in 2-chlorobenzaldehyde **10**, which has a strong, very attractive, fruity note recalling the tagetes oil with undertones of dried fruit.

The incorporation of an aldehyde group in the 'natural' pattern of the α- and β-ionones completely changed the odour of **7a**–**b**. We, at the moment, cannot state whether this is due to the concomitant presence of chlorine. On the other hand, the substitution of that chlorine with a dialkylamino group gave **8a**–**b**, providing a pleasant and well-balanced note in which the 'medicinal', tarry and bitter notes disappeared.

The presence of chlorine together with an aldehyde group seems to have played well in the p-menthane pattern. In fact **9** showed a note, which is not comparable to other existing notes and whose strength relies on the concentration; while **10**, on the other hand, which with only one extra double bond becomes aromatic, possesses a characteristic fragrance which could well apply to both flowery and fruity compositions.

Table I. Olfactory properties of compounds **7**–**11**.

Compound	[a]Strength	Evolution	Persistence	P. after 24 h	Quality	Potential use
7a	5	long lasting	good	sufficient	Harsh, bitter, tarry, unpleasant	Special use (e.g. tarry shampoo). Fixative
7b	4	long lasting	medium	sufficient	Penetrating, sharp, similar to β-ionone but more woody and medicinal	Pharmaceutical use. Top note.
8a	2	long lasting	good	low	Powdery, vanillic, a reminder of orris oil	Lipsticks, powder cosmetics. Fixative
8b	2	towards saffron note	good	low	Fruity, cooked prunes, a reminder of tagetes oil	Top or body note for fruity or spicy compositions
9	6	towards Quinoline note	good	good	Bitter, woody	Not comparable to existing notes. According to concentration can be used as top, body or end note
10	4	towards fruity notes	good	adequate	Flowery, fruity, a reminder of dried fruits	Flowery and fruity compositions. Top or body note.
11					Too weak for an evaluation	

[a] 1 = very weak, 2 = weak, 3 = moderate, 4 = strong, 5 = very strong, 6 = extremely strong. The olfactory evaluation was carried out on pure compounds and on the same compounds diluted in ethanol 1 to 10.

4.2. Cytotoxicity

Compounds **9** and **10** were selected for a preliminary study of cytotoxicity and the results obtained by means of three different in vitro assays are reported as dose–response curves in *figure 8*. Table II shows the IC_{50} (concentration inhibiting viability by 50%) obtained from those curves. All three assays gave curves having very similar patterns. It is evident that, at low concentrations, **10** is much less cytotoxic than **9**, whereas the opposite is true at concentrations higher than 5 mM. Moreover, by comparing the IC_{50}s (*table II*) it is clear that **10** has an intrinsic cytotoxicity which is one order of magnitude lower than that of **9**. Additionally, **9** has the same IC_{50} of SDS, therefore this compound should be considered as a strong irritant. Nonetheless, the IC_{10}s (concentration inhibiting viability by 10%) of **9** and SDS (*table II*) demonstrate that, at micromolar concentrations, only 10% of the cells die.

Therefore, this in vitro evaluation suggests that **10** is safer than **9**, and **9** should be used in formulations at low

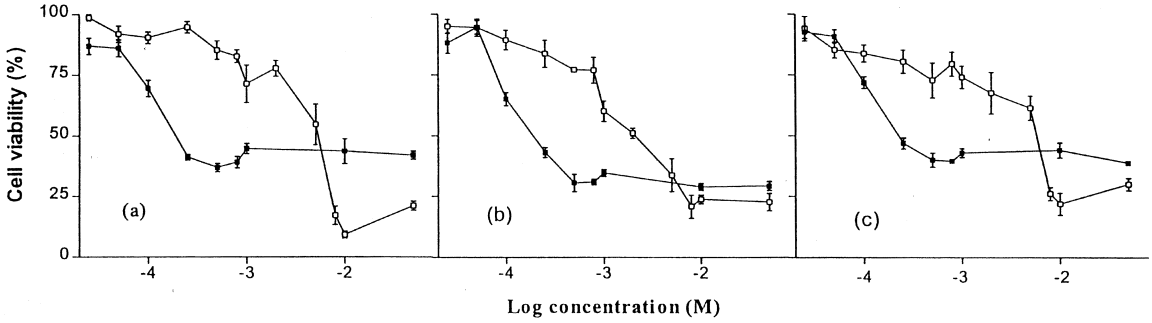

Figure 8. Comparative cytotoxicity of compound **9** (■) and **10** (□) in NCTC 2544 human keratinocytes as detected with (a) NRU, (b) MTT, (c) TPC assays. The values represent the mean ± SEM of the four experiments in quadruplicate.

Table II. Cytotoxicity of **9**, **10** and sodium dodecylsulphate (SDS) in NCTC 2544 human keratinocytes evaluated as IC_{50} and IC_{10}.

	IC_{50} (mM)		
Compound	NRU test	MTT test	TPC test
9	0.123	0.12	0.119
10	3.2	1.5	2.5
SDS	0.132	0.133	0.133
	IC_{10} (mM)		
9	0.022	0.03	0.05
10	0.1	0.1	0.038
SDS	0.07	0.047	0.103

concentrations. However, conditions of use, chemical and physical properties and levels of absorption should also be taken into consideration.

5. Conclusion

In summary, the introduction of a formyl group, even if accompanied by chlorine or a dialkylammino group, has once more proven to furnish odorants with unusual notes that could stimulate new creations in perfumery and aromatisation.

Regarding the cytotoxicity tests, we thought it interesting to evaluate the cytotoxicity of our molecule by means of three in vitro assays that test different endpoints in order to assess its toxic potential in a non-animal system. It is clear that these tests cannot replace the complete safety appraisal of a cosmetic ingredient, but they give an indication of the degree of potential toxicity for further in vivo investigations or more specific in vitro tests (i.e. contact irritancy and hypersensitivity).

6. Experimental protocols

6.1. Chemistry

Melting points were determined with Fisher-Johns apparatus and are uncorrected. The IR spectra were recorded in chloroform or in potassium bromide disks on a Perkin-Elmer 398 spectrometer. The ^{1}H- and ^{13}C-NMR spectra were recorded on a Varian Gemini 200 (200 MHz, ^{1}H; 50 MHz, ^{13}C) spectrometer in deuteriochloroform solutions with tetramethylsilane as the internal standard ($\delta = 0$). The purity of all compounds was checked by thin-layer chromatography on silica gel 60-F-254 precoated plates and the spots were located in UV light or by vanillin in sulfuric acid. Elemental analyses were performed on a Carlo Erba 1106 Elemental Analyser in the Microanalysis Laboratory in our Institute and the results were within ± 0.4% of theoretical values. For spectral data of **7**–**8** see [7].

6.1.1. Vilsmeier reaction on α- and β-ionones. General procedure

Phosphorous oxychloride (50.0 mmol; 4.57 mL) was added dropwise, within 15 min at 0 °C, to 3.87 mL of N,N-dimethyl(diethyl)formamide in a two-necked flask protected from atmospheric moisture and efficiently stirred with a magnetic bar. A solution of α/β-ionones (25.0 mmol) in 3 mL of dimethylformamide was dropped into the above Vilsmeier reagent, cooled at –20 °C, left to stir while the temperature was left to rise to 0 °C for a total of 45 min and finally poured onto crushed ice. The aqueous layer was separated and treated as further described for each compound.

6.1.1.1. 3-Chloro-5-(2,6,6-trimethyl-2-cyclohexen-1-yl)-2,4-pentadienal **7a**

The aqueous layer was carefully neutralized with 10% sodium hydroxide (pH control) and allowed to stand overnight at room temperature. The colourless oil, which was easily separated out from the aqueous phase, was purified by column chromatography on silica gel, eluting with toluene, giving **7a** in 25% yield. IR (film): ν 2 900, 2 840, 1 665, 1 615, 1 575, 1 160 cm^{-1} [7].

6.1.1.2. 3-Chloro-5-(2,6,6-trimethyl-1-cyclohexen-1-yl)-2,4-pentadienal **7b**

By using the above procedure, compound **7b** was finally purified by column chromatography as a colourless oil in 28% yield. IR (film): ν 2 920, 2 860, 1 670, 1 610, 1 590, 1 570, 1 170 cm^{-1} [7].

6.1.1.3. 3-Dimethylamino-5-(2,6,6-trimethyl-2-cyclohexen-1-yl)-2,4-pentadienal **8a**

The aqueous layer was alkalinized with 30% sodium hydroxide and allowed to stand overnight at room temperature. The resulting water/oil mixture was extracted with chloroform, and the combined organic layer dried on magnesium sulphate and evaporated to give an oil which solidified on standing, giving **8a** as an already pure pale yellow powder in 40% yield. m.p. 147 °C from n-hexane [7].

6.1.1.4. 3-Diethylamino-5-(2,6,6-trimethyl-2-cyclohexen-1-yl)-2,4-pentadienal **8b**

The aqueous layer, worked as above, gave a thick oil which was purified by column chromatography on silica gel, eluting with ethyl acetate, giving **8b** as a thick yellow oil in 28% yield. IR (film): ν 2 900, 1 620, 1 540, 1 430, 1 380, 1 310, 1 260, 1 200, 1 180 cm^{-1} [7].

6.1.2. Vilsmeier reaction on Carvone. General procedure

A solution of 10 g (66.6 mmol) of Carvone in 5 mL of dimethylformamide (DMF) was added at 20 °C over 5 min to the Vilsmeier reagent, which was prepared by adding phosphorus oxychloride (6.04 mL; 66.6 mmol) over 5 min to a stirring 5.10 mL (66.6 mmol) of DMF at 0 °C and allowing the mixture to continue stirring at 20 °C for 20 min. The mixture of Carvone and Vilsmeier reagent was stirred at 20 °C from 1–3.5 h, crushed ice was then added and stirred for an additional 20 min. The aqueous layer was separated and basified with a solution of sodium hydroxide and stirred overnight. The red oil, which separated during the night, was extracted with chloroform (3 × 60 mL) and the combined organic layers were washed with water (2 × 70 mL), dried over anhydrous magnesium sulfate and evaporated to give a thick dark red oil. Separation by column chromatography on silica gel (200 g) using toluene, toluene/ethyl acetate (1:1) and ethyl acetate as eluents gave **9**, **10** and **11**. Toluene provided compounds **9** and **10**, toluene/ethyl acetate gave the unreacted Carvone and ethyl acetate afforded indene derivative **11**. Elution with ethanol gave a thick brownish unworkable oil.

6.1.2.1. 2-Chloro-6-isopropenyl-3-methyl-cyclohexa-1,3-dienecarbaldehyde **9**

By use of the general procedure, the reaction mixture was allowed to stir for 2 h at 20 °C. Separation by column chromatography on silica gel of the final thick dark red oil gave **9** in 18% yield as yellow oil. IR (film) ν 2 910, 1 660, 1 550, 890 cm^{-1}; ^{1}H-NMR (200 MHz, $CDCl_3$): δ 1.72 (3H, s, CH_3), 1.97 (3H, s, CH_3), 2.46 (2H, m, CH_2), 3.45 (1H, dd, CH), 4.59 (1H, s, CH_2), 4.74 (1H, s, CH_2), 6.24 (1H, m, CH), 10.20 (1H, s, CHO); ^{13}C-NMR (50 MHz, $CDCl_3$) 19.61 (CH_3), 21.63 (CH_3), 27.71 (CH_2), 37.59 (CH), 111.83 (CH_2), 132.05 (C), 132.61 (C), 132.83 (CH), 143.37 (C), 149.28 (C), 190.75 (CHO).

6.1.2.2. 2-Chloro-6-isopropenyl-3-methyl-benzaldehyde **10**

The final thick dark red oil, obtained after 3 h of reaction time, and chromatographed on silica gel eluting with toluene provided **10** in 14% yield as yellow oil. IR (film) ν 2 910, 1 660, 1 600, 1 570, 1 440, 1 360 cm^{-1}; ^{1}H-NMR (200 MHz, $CDCl_3$) 2.06 (3H, s, CH_3), 2.42 (3H, s, CH_3), 4.83 (1H, s, CH_2), 5.24 (1H, s, CH_2), 7.09 (1H, d, CH, J = 7.72 Hz), 7.36 (1H, d, CH, J = 7.72 Hz) 10.40 (1H, s, CHO); ^{13}C-NMR (50 MHz, $CDCl_3$) 20.52 (CH_3), 24.92 (CH_3), 117.26 (CH_2), 127.81 (CH), 131.85 (C), 134.87 (CH), 135.96 (C), 137.40 (C), 144.30 (C), 146.12 (C), 192.24 (CHO).

6.1.2.3. 7-Chloro-3,6-dimethyl-1H-indene-2-carbaldehyde **11**

By use of the general method, the reaction mixture was allowed to stir for 3.5 h at 20 °C. The final thick dark red oil was separated by column chromatography on silica gel. The elution with ethyl acetate afforded **11** in 25% yield as yellow crystals. M.p. 165 °C from cyclohexane. IR (KBr) ν 2 910, 1 640, 1 600, 1 440 cm^{-1}; ^{1}H-NMR (200 MHz, $CDCl_3$) 2.46 (3H, s, CH_3), 2.53 (3H, s, CH_3), 3.64 (2H, s, CH_2), 7.30 (1H, d, CH, J = 7.7 Hz), 7.33 (1H, d, CH, J = 7.7 Hz), 10.20 (1H, s, CHO); ^{13}C-NMR (50 MHz, $CDCl_3$) 11.43 (CH_3), 20.53 (CH_3), 36.39 (CH_2), 120.20 (CH), 130.43 (CH), 131.45 (C), 137.72 (C), 139.66 (C), 143.25 (C), 144.43 (C), 155.76 (C), 187.32 (CHO).

6.2. Biological methods

6.2.1. Chemicals

Dulbecco's modified Eagle medium (DMEM), (MTT) tetrazolium salt, sodium dodecylsulphate (SDS) were purchased from Sigma (St Louis, MO, USA); L-glutamine and neutral red were from ICN Biomedicals Inc. (Costa Mesa, CA, USA); Trypsine-EDTA was fom Gibco BRL (Paisley, Scotland); foetal calf serum was from Mascia Brunelli (Milan, Italy); the protein assay dye reagent was from BioRad Laboratories GmbH (Munchen, Germany); cell culture flasks and 96-well plates were from Costar (Cambridge, UK).

6.2.2. Cell cultures

Normal human keratinocyte cell line NCTC 2544 (kindly furnished by Prof. Marinovich, Institute of Pharmacological Sciences, Milan, Italy) grown in DMEM medium supplemented with 10% foetal calf serum and 2% L-glutamine at 37 °C in a humidified atmosphere of 5% CO_2/95% air.

The medium was changed every 2–3 days and, when the original flask was approximately 80% confluent, the cells were subcultured by trypsin-EDTA digestion.

6.2.3. Treatments

Cells were seeded 24 h before treatment in 96-well plates at 4×10^4 cells/well (keratinocytes) in order to obtain semi-confluent cultures. Cells were exposed for 3 h to the medium containing various concentrations (0.025–50 mM) of **9** and **10** dissolved in 1.25% dimethylsulphoxide (final concentration, DMSO). Positive controls received SDS (0.01–0.2 mM) and controls for solvent were carried out. After that time, the medium was removed and the plates were prepared for the NRU, MTT and TPC assays.

6.2.4. Neutral red uptake

Cells were exposed to a medium containing 50 µg/mL of neutral red dye and processed according to the Borenfreund et al. method [14]. After 3 h incubation at 37 °C, the cells were washed twice in PBS and fixed according to the procedure described by Riddell et al. [15]. The plates were then left at room temperature for 10 min and the absorbance of the extracted neutral red dye was read at 550 nm wavelength in a Uniskan II microplate reader (Labsystems, Helsinki, Finland).

6.2.5. MTT tetrazolium assay

MTT was dissolved in PBS (5 mg/mL) and given to the cells (10 µL/mL medium), according to the Mosmann method [16]. After 3 h incubation at 37 °C, a solution of 1 N hydrogen chloride/isopropanol (1:24; v/v) was added to each well and mixed to dissolve the formazan dark blue crystals. After a few minutes at room temperature the plates were read at 550 nm wavelength.

6.2.6. TPC assay

Total cellular protein content was quantified using Coomassie blue dye that is taken up only by fixed cells in the monolayer, according to a slight modification of the method of Bradford [12]. Plates were washed twice with PBS after the NRU assays and 100 µL NaOH were added to each well for 1 h at 37 °C to dissolve the protein. After dilution of the samples, the Bio-Rad reagent was added and the absorbance was read at a wavelength of 595 nm.

6.2.7. Statistical analysis

All experiments were performed at least four times using four wells for each concentration of the tested agents. Results were expressed as percentage of viability compared to control. Linear regression analysis was used to compute the concentration needed to reduce absorbance to 50% (IC_{50}).

Acknowledgements

We wish to thank Dr Luigi Rigano (via Calabria 5-20158 Milan) for the olfactory evaluations. Financial support was provide by CNR and MURST (Rome).

References

[1] Markert T., Henkel K.-G.a.A., Fed. Rep. Ger., DE 1981 7042-4, 1999 (Chem. Abstr. 131:276818-20).

[2] Chalk A.J., Virgilio J.A., Givaudan-Roure (International) S.A., EP 952139, 1999 (Chem. Abstr. 131:299148).

[3] Sanders J.M., Taylor W.I., Hill I.D., Kryschuk J.J., International Flavors and Fragrances Inc., US 4031161, 1977 (Chem. Abstr. 87:151775).

[4] Hagen J., Klaus B., Henkel K.-G.a.A., Fed. Rep. Ger., DE 2849642, 1978 (Chem. Abstr. 94:15287), and references cited therein.

[5] Jegou E., Polonsky J., Lederer E., Schulte-Elte K.H., Egger B., Ohloff G., Nouv. J. Chim. 1 (6) (1977) 529–531.

[6] Marson C.M., Giles P.R. (Eds.), Synthesis using Vilsmeier reagents, CRC Press Inc., Boca Raton, 1994.

[7] Sottofattori E., Anzaldi M., Balbi A., J. Heterocycl. Chem. 35 (1998) 1377–1380.

[8] Anzaldi M., Sottofattori E., Rizzetto R., Granello di Casaleto B., Balbi A., Eur. J. Med. Chem. 34 (1999) 837–842.

[9] Dauphin G., Synthesis (1979) 799–801.

[10] Pani M., Mugnoli A., Sottofattori E., Acta Crystallographica C56 (2000) 840–842.

[11] Viviani B., Marinovich M., Galli C.L., ATLA 20 (1992) 108–115.

[12] Bahich H., Borenfreund E., ATLA 18 (1990) 129–144.

[13] Bradford M.M., Anal. Biochem. 72 (1976) 248–257.

[14] Borenfreund E., Babich H., Martin-Alguacil N., Toxicol. In Vitro 2 (1988) 1–6.

[15] Riddell R.J., Clothier R.H., Balls M., Food Chem. Toxicol. 24 (1986) 469–471.

[16] Mosmann T.J., Immunol. Methods 65 (1983) 55–63.