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The photoreactions of simple amides with NO. Gaining insight into radical bio-damages through an EPR case study

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ABSTRACT

Eight simple amides have been subjected to UV irradiation in the presence of either MNP or NO. In all cases radical species were generated: these were detected by means of EPR spectroscopy in the form of different nitroxides resulting from the trapping of the primary radicals. NO acted as a double spin trap, scavenging a radical to afford a diamagnetic nitroso derivative that in turn acted as trap towards another radical unit. As amido-groups are present in components of skin tissue and may be present in many therapeutic or cosmetic products used as skin sunscreen, and NO is a ubiquitous endogenous reactive species, the nitroxides detected in the present studies might participate in radical processes triggered by sun exposure and resulting in damages, even severe, of biological tissues.

1. Introduction

The effects of UV radiation on the skin have been described, but only the first reaction-step of the 'chemistry' underneath the induced skin sensitization due to endogenous or exogenous stimulus seems to have been ascertained, whilst the molecular mechanisms by which secondary effects are produced have not been clarified. Indeed, photo-excited states of endogenous UVA chromophores, such as porphyrins, melanin precursors, and crosslink-fluorophores of skin collagen can exert a negative action, giving rise to reactive oxygen species, ROS, by direct reaction with substrate molecules (type I photosensitization) or with molecular oxygen (type II). Some of these species, including alkoxy, peroxy radicals and the superoxide anion, are actually involved in the first step of the process induced by UVA, and have been recognized as being responsible for skin damage.[1,2]

The different amido-groups belonging to the peptides and ceramides present in the epidermis, are among the many functional groups liable to generate radicals following UV irradiation; in fact, the photoexcitation of the amido-group can lead to the formation of both carbon centered and nitrogen centered radicals. Oxygen centered radicals can also be formed by further reaction of the carbon centered species with oxygen, while the oxygen end of the photoexcited triplet carbonyl group may act as an alkoxy radical.

In addition to the ROS and the above mentioned radicals, other reactive species participate in chemical processes taking place in human body, and among these NO, a ubiquitous and endogenously formed radical has a major relevance. In particular, nitric oxide plays a key role in the dermal response to external stimuli: it is present in lesional psoriatic skin, in squamous cell carcinomas, during wound healing and contributes to the formation of sunburn erythema.[3] On the favorable side, NO is an effective inhibitor of lipid peroxidation and its coordinated action on gene expression and preservation of membrane function effectively protects against UV-A or ROS induced apoptotic and necrotic cell death.[4]

Significant quantities of NO are continuously released from human skin[5] and it has been repeatedly shown that the NO-synthase dependent production of NO potentially occurs in all dermal cell types.[4] Nitric oxide is also produced at the skin surface by bacterial and chemical reduction of sweat nitrate.[5] In addition, it has also been proved that NO release is significantly increased following exposure to UV radiation, as in the case of sun exposure.[5,6]

The most reliable mechanisms of skin damage are considered to involve radical intermediates: thus, the detection and identification of these species would greatly help the understanding of the mechanism of interaction. EPR spectroscopy, especially when used in combination with the spin trapping technique, has long since been recognized as the most powerful tool for the detection and identification of free radicals, normally very short lived intermediates. Its application to the study of processes taking place

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even in a very complex 'reaction pot' such as the skin has been largely exploited either directly, especially through the use of nitrones (e.g., 5,5-dimethyl-1-pyroline *N*-oxide, DMPO, and *N*-*tert*-butyl-α-phenyl nitrone, PBN) able to trap carbon or oxygen centered radicals forming detectable EPR nitroxides, or indirectly monitoring the decay of nitroxidic spin probes such as 2,2,6,6-tetramethylpiperidine 1-oxyl (TEMPO), 3-carbamoyl-2,2,5,5-tetramethylpyrrolidine-1-oxyl (PCM), and 3-carboxy-2,2,5,5-tetramethylpyrrolidine-1-oxyl (PCA) to show the presence of ROS, which are known to convert nitroxides to EPR silent hydroxylamines. Unfortunately, the results were not always unequivocal. In particular, the finding that in several cases the decay of the EPR signal of the nitroxides could only be observed in the presence of thiols[7–10] or of NADH,[11] made the actual formation of ROS uncertain, let alone their direct interaction with the spin probes.

On the other hand, the presence of ROS and other radicals along with that of nitric oxide may result in adventitious radical species. First NO, being a radical itself, might scavenge other paramagnetic species (carbon centered radicals or ROS) to give diamagnetic nitroso derivative. In a second stage, these would act as spin traps leading to the formation of EPR detectable nitroxides.

Following this hypothesis, we conducted a case study on the photoexcitation of amides **1**–**8** in the presence of NO, not so much to isolate the resulting products but with the aim to identify the newly formed radicals that might model those formed in the actual biological processes. As a preliminary support of this study the photoexcitation of the same amides in the presence of MNP, a well-known spin trap, was at first carried out. Several nitroxides, deriving from the trapping of radicals originating from both the β-cleavage and intermolecular hydrogen abstraction of the excited carbonyl group, were identified and compared with those detected using the nitric oxide as trap.

2. Results

Photolysis of ACN solutions of alkyl amides **1**–**8** was expected to lead to several radicals deriving from β-fragmentation (acyl radicals, alkyl radicals, and aminyl radicals) and from intermolecular hydrogen abstraction by the photoexcited carbonyl group. None of these 'primary' species were directly detected when the amides were photolyzed, but in the presence of MNP, the corresponding adducts, whose spectral parameters are collected in Table 1, were observed. In nearly all cases, signals due to di-*tert*-butyl nitroxide, a species whose formation is practically unavoidable when photolyzing MNP, were also detected.

In the experiments run in the presence of PILA 124 (2-ethoxythioxanthone) and irradiating with light at $\lambda \geq 400$ nm, only MNP adducts of the radicals originating via hydrogen abstraction from the amides by the photoexcited PILA 124 were observed.

The 'primary' radicals could also not be directly detected upon irradiation of NO-saturated solutions of amides **1**–**8**, which instead resulted again in the formation of nitroxides (Table 1).

2.1. *N,N*-Dimethylformamide, 1

Photolysis of **1** in the presence of MNP only led to the detection of the spectrum from a nitroxide, identified as **1a**, overimposed to that of di-*tert*-butyl nitroxide. The spectral parameters indicate that **1a** results from the trapping by MNP of the radical originating via an intermolecular hydrogen abstraction from a methyl group by a photoexcited carbonyl.

The failure to show any trace of *tert*-butyl-acetyl nitroxide suggests that at the temperature of operation, hydrogen abstraction by the triplet carbonyl prevails over β-fragmentation processes. The identification of **1a** was further substantiated by the finding that an identical spectrum was observed when irradiating ($\lambda \geq 400$ nm) the solution after adding PILA 124. Under these conditions PILA 124 abstracts one hydrogen from a methyl group of **1** through its excited carbonyl group leading again to **1a**.

Room temperature photolysis of **1** in the presence of NO, led to the EPR detection of only one radical species that was identified as nitroxide **1b**, its spectral parameters being consistent with those reported in the literature.[12] Although unexpected, the detection of nitroxide **1b** can be justified on the basis of the two competing photocleavage paths of dimethylformamide; that is cleavage of the C(O)–N bond and cleavage of an *N*-methyl bond.[13]

Thus, it may be tentatively envisaged a C–N β-fragmentation of photoexcited **1**, followed by coupling of the resulting formyl radical with nitric oxide to give HC(O)NO. This in turn might trap a methyl radical from the other type of fragmentation of DMF.

2.2. *N*-Methylacetamide, 2

In contrast with **1**, photolysis of *N*-methylacetamide **2** in the presence of MNP led to the observation of three distinct radical species originating via either hydrogen abstraction by the photoexcited carbonyl (radicals **2a** and **2b**) or its β-scission (radical **2c**). While nitroxide **2a** can be assimilated to **1a**, that analogous to **2b** was not observed with DMF, **1**. Even if some doubts could be reasonably cast on the identity of **2b** as di-*tert*-butyl nitroxide, it exhibits a very similar spectrum, and we base its identification on the value of the nitrogen splitting constants, which were found to vary slightly but definitely with every amide (see below) being somewhat larger than that normally observed for di-*tert*-butyl nitroxide in acetonitrile. In principle one might envisage the authentic **2b** via photoreaction of *N*-methylethanolamine with di-*tert*-butyl peroxide in the presence of MNP. On the other hand, the necessary starting amine is commercially unavailable and we believe that it synthesis would not be worth the effort as its reaction with *tert*-butoxy radicals may result in the formation of several different radicals and hence in complex and uninformative spectra. The identification of **2b** is therefore to be considered tentative.

In principle, doubts might be also cast on the identity of **2c**, as the β-scission of photoexcited **2** may result in the formation of both a formyl and a dimethylaminyl radical or a methyl and a dimethylaminocarbonyl radical. We favor the former kind of β-scission, as if the latter took place, the methyl radical should be readily trapped by MNP. On the other hand, MNP is much less efficient in trapping aminyls than alkyl radicals, and the failure to detect *tert*-butyl dimethylamino nitroxide is not surprising. In any case, radical **2c** disappeared when PILA 124 was added to the solution, leaving nitroxides **2a** and **2b** as the only detectable species.

The prolonged photolysis of a NO saturated ACN solution of **2** led to a strong clean signal from a radical identified as **2d** (see Fig. 1); yet, it may be worth noting that while the formation of this species clearly involves two radicals from a hydrogen-abstraction process, no *tert*-alkyl nitroxide involving their counterpart was observed.

2.3. *N,N*-Dimethylacetamide, 3

Competition between β-scission and hydrogen abstraction was also evident when photolyzing **3** in the presence of MNP. In this case *tert*-butyl acetyl nitroxide **2c** could be readily detected.

This radical, however, was accompanied by two additional MNP adducts, both exhibiting interaction of the unpaired electron with a nitrogen and two equivalent hydrogen atoms. These were identified as the isomeric nitroxides **3a** and **3b** on the basis of the similarity of their spectral parameters with those exhibited by **1a** and **2a**. Geometrical isomerism in amides is well established and a large number of NMR studies have addressed this issue.[14] It would then appear that a photoexcited amide abstracts a hydrogen from

Table 1
Radicals upon photolysis of amides **1**–**8**[a]

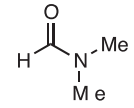
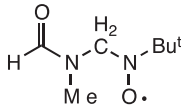
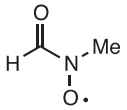
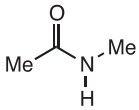
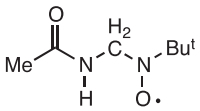
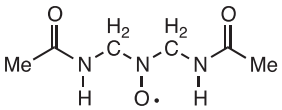
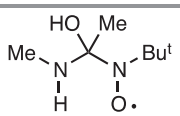
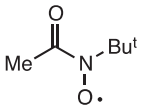
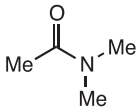
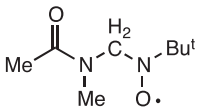
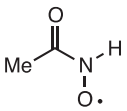
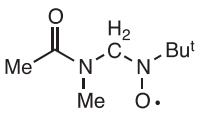
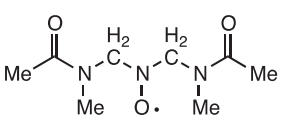
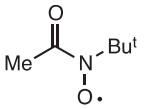
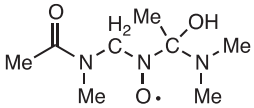
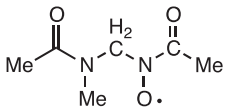
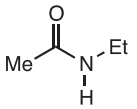
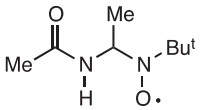
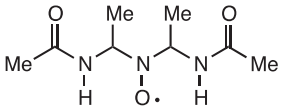
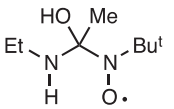
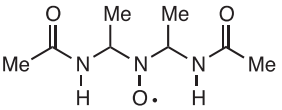
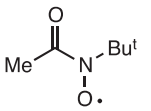
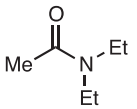
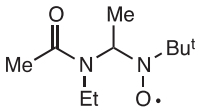
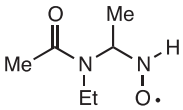
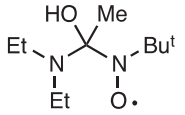
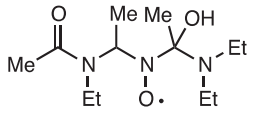
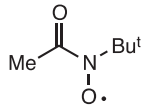
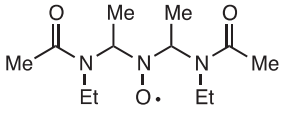
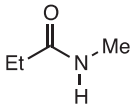
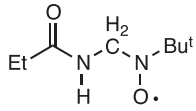
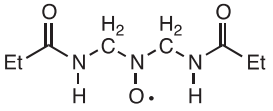
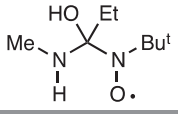
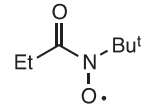
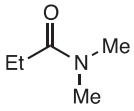
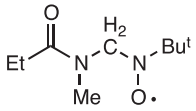
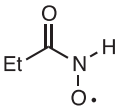
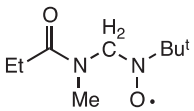
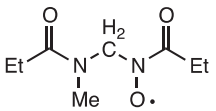
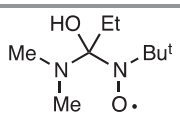
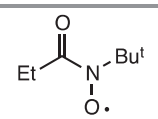
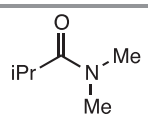
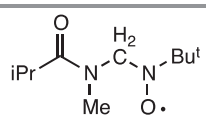
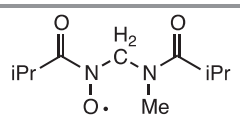
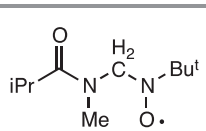
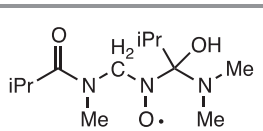
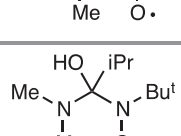
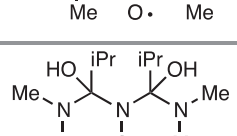
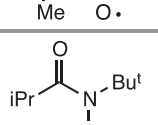
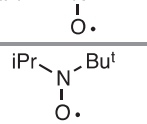
In the presence of **MNP**				In the presence of **NO**		
Substrate	Radical	EPR Spectral Parameters	T/°K	Radical	EPR Spectral Parameters	T/°K
 **1**	 **1a**	g =2.0060$_7$ $a_{(H+H')}$=1.75 a_N = 0.234 a_N = 1.465	263	 **1b**	g =2.0068$_9$ a_H = 0.137 a_{3H} = 0.836 a_N = 0.670	293
 **2**	 **2a**	g =2.0061$_1$ a_{2H} = 0.796 a_N = 0.267 a_N = 1.513	263	 **2d**	g =2.0061$_2$ a_{4H} = 0.621 a_{2N} =0.261 a_N = 1.443	243
	 **2b**	g =2.0059$_4$ a_N = 1.594	223			
	 **2c**	g =2.0069$_2$ a_N = 0.792	223			
 **3**	 **3a**	g =2.0061$_2$ a_{2H} = 0.801 a_N = 0.293 a_N =1.484	263	 **3c**	g =2.0070$_5$ a_H = 1.070 a_N = 0.630	293
	 **3b**	g =2.0061$_2$ a_{2H} = 1.138 a_N = 0.16 a_N = 1.520	263	 **3d**	g =2.0060$_2$ $a_{2H\alpha}$ =0.229 $a_{2H\beta}$ =1.949 a_{2N} = 0.226 a_N = 1.484	223
	 **2c**	g =2.0069$_2$ a_N = 0.782	263	 **3e**	g =2.0063$_5$ a_H = 0.202 a_H = 2.146 a_N = 0.229 a_N = 1.494	223
				 **3f**	g =2.0066$_0$ a_{2H} = 0.623 a_N = 0.112 a_N = 0.753	223
 **4**	 **4a**	g =2.0057$_2$ a_H = 0.130 a_N = 0.237 a_N = 1.496	263	 **4c**	g =2.0059$_0$ a_{2H} =0.570 a_{2N} =0.204 a_N = 1.475	293
	 **4b**	g =2.0057$_7$ a_N = 1.572	263	 **4d**	g =2.0058$_5$ a_{2H} =0.268 a_{2N} =0.250 a_N = 1.420	293
	 **2c**	g =2.0069$_2$ a_N = 0.792	263			
 **5**	 **5a**	g =2.0059$_8$ a_H = 0.386 a_N = 0.427 a_N = 1.450	263	 **5c**	g =2.0060$_4$ a_H = 0.307 a_N = 0.250 a_H = 1.360 a_N = 1.432	293

Table 1 (*continued*)

	 **5b**	g =2.0059$_0$ a_N = 1.573	263	 **5d**	g =2.0060$_6$ a_H = 0.248 a_N = 0.164 a_N = 1.380	293
	 **2c**	g =2.0066$_9$ a_N= 0.792	263	 **5e**	g =2.0060$_2$ a_{2H} =0.919 a_{2N} =0.277 a_N = 1.384	293
 **6**	 **6a**	g =2.0058$_9$ a_{2H} = 0.787 a_N = 0.268 a_N = 1.512	298	 **6d**	g =2.0058$_9$ a_{4H} = 0.807 a_{2N} = 0.258 a_N = 1.512	298
	 **6b**	g =2.0058$_9$ a_N = 1.568	298			
	 **6c**	g =2.0066$_7$ a_N = 0.807	298			
 **7**	 **7a**	g =2.0058$_9$ a_{2H} = 0.786 a_N = 0.322 a_N = 1.486	273	 **7d**	g =2.0071$_0$ a_H = 1.089 a_N = 0.630	298
	 **7b**	g =2.0058$_9$ a_{2H} = 1.175 a_N = 0.140 a_N = 1.525	273	 **7e**	g =2.0065$_4$ a_{2H} = 0.617 a_N = 0.111 a_N = 0.750	223
	 **7c**	g =2.0058$_8$ a_N = 1.573	273			
	 **6c**	g =2.0066$_7$ a_N = 0.801	273			
 **8**	 **8a**	g =2.0059$_2$ a_{2H} = 0.805 a_N = 0.314 a_N = 1.512	243	 **8f**	g =2.0065$_7$ a_{2H} = 0.678 a_N = 0.098 a_N = 0.762	233
	 **8b**	g =2.0059$_2$ a_{2H} = 1.159 a_N = 0.147 a_N = 1.521	243	 **8g**	g =2.0065$_7$ a_H = 2.040 $a_H \approx$ 0.00 a_N = 0.200 a_N = 1.480	223
	 **8c**	g =2.0059$_9$ a_N = 1.575	293	 **8h**	g =2.0062$_5$ a_N = 1.422	223
	 **8d**	g =2.0066$_2$ a_N = 0.801	293			
	 **8e**	g =2.0059$_0$ a_H = 0.183 a_N = 1.535	293			

[a] Hyperfine splitting constants in mT.

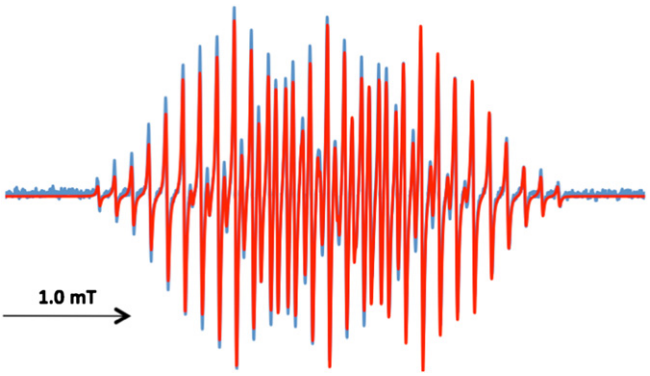


Fig. 1. EPR spectrum observed upon photolysis of a NO saturated ACN solution of 2 at 243 K. Blue: experimental; red: computer simulation.

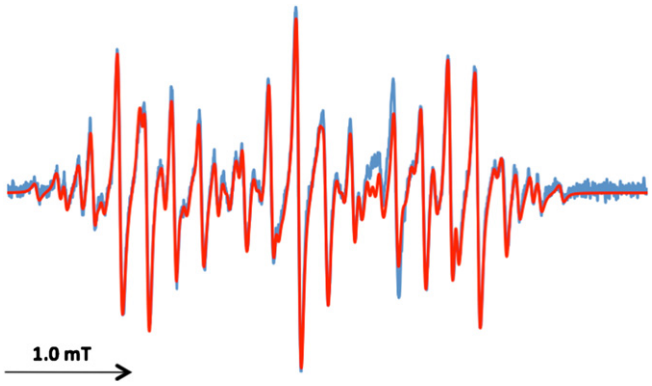


Fig. 2. EPR spectrum observed upon photolysis of a NO saturated ACN solution of compound **4** at 233 K. Blue: experimental; red: computer simulation.

either of the magnetically non-equivalent methyl groups of other amide molecules leading to two 'different' primary-alkylamino radicals that, once trapped by MNP, afford **3a** and **3b**. Also in the case of **3**, no *tert*-butyl *tert*-alkyl nitroxide was observed, originating from the trapping of the counterpart of the primary-alkylamino radicals leading to **3a** and **3b**. The main difference to be found in the spectral parameters of these two nitroxides concerns the couplings of the β-hydrogen and β-nitrogen atoms that, due to their position, are more conformation sensitive.

As had been the case with amides **1** and **2**, the addition of PILA 124 totally suppresses β-fragmentation, resulting in the complete disappearance of the acyl nitroxide **2c**.

Hydrogen abstraction and β-scission were also operative upon photolysis of solutions of **3** and NO: spectra showed the presence of four different nitroxides. Nitroxide **3d**, results from the coupling of NO with the primary alkylamino radical (hydrogen abstraction from an *N*-methyl group) followed by trapping of an identical radical by the resulting alkylnitroso derivative. The same alkylnitroso derivative also traps the other *tert*-alkylamino radical formed in the hydrogen abstraction to give nitroxide **3e**, whereas acyl nitroxide **3f** is formed when yet the same alkylnitroso derivative traps the acetyl radical resulting from β-scission. It is worth noting that the four β-hydrogen atoms in nitroxides **3d** are magnetically equivalent only in pairs and that the two β-hydrogens in **3e** are not equivalent, while in nitroxide **2d** all the four β-hydrogen atoms are equivalent. It would then appear that the replacement of the amidic hydrogen with a second methyl group hampers the rotation about the N(O·)—C bonds. Yet, in radical **3f**, where a less sterically demanding acetyl unit replaces one alkylamido moiety, the two methylenic hydrogen atoms are again equivalent.

The spectral parameters determined for the fourth nitroxide detected when photolyzing **3** and NO led to its identification as the acetyl nitroxide **3c**. Its formation is an unexpected finding, fairly intriguing to be accounted for (see Discussion).

2.4. *N*-Ethylacetamide, 4

Only species deriving from a hydrogen abstraction process were observed when photolyzing a solution of **4** containing some MNP and PILA 124. These radicals could be identified as the *tert*-butyl secondary-alkylamino nitroxide **4a** and the *tert*-butyl *tert*-alkylamino nitroxide **4b**. As expected, in the absence of PILA 124 the acyl nitroxide **2c** could also be observed.

The photoreaction of **4** with NO led to the detection of an EPR spectrum due to the superimposition of the signals from two nitroxides exhibiting different couplings with the same groups of atoms, that is, from two isomeric species (see Fig. 2).

As both species showed, beside that with the nitroxidic nitrogen, the interaction of the unpaired electron with two equivalent nitrogen and two equivalent hydrogen atoms, to both were assigned MeC(O)NHCH(Me)N(O·)CH(Me)NHC(O)Me as the same general structure, what may be consistent with the presence of two diasteroisomers (**4c** and **4d**). Indeed, over the investigated temperature range, only the splittings of the two equivalent hydrogens experienced an appreciable variation increasing from 0.194 mT at 233 K to 0.268 mT at 293 K for one species and from 0.539 mT at 233 K to 0.570 mT at 293 K for the other, while all other parameters only experienced negligible variations. As in the corresponding nitroxide from **2** only one isomer, i.e., **2d**, was detected, it seems reasonable attributing this conformational behavior to the presence of the more sterically demanding ethyl group in **4** than that of a methyl group in **2**.

2.5. *N,N*-Diethylacetamide, 5

Photolysis of **5** in the presence of MNP led to the detection of three different nitroxides. The acetyl *tert*-butyl nitroxide **2c**, which derives from β-fragmentation of photoexcited **5**, the nitroxides resulting from the trapping by MNP of the secondary-alkyl (**5a**), and tertiary-alkyl radical (**5b**) generated via intermolecular hydrogen abstraction by photoexcited **5**. Consistently, addition of PILA 124 to the solution being photolyzed resulted in the disappearance of nitroxide **2c** and in a significant increase of the intensity of the signal from **5a**.

When photolyzed in the presence of NO, **5** led to the detection of very complex spectra with a fairly significant dependence on temperature. We interpreted the spectrum recorded at room temperature as the sum of the signals from the three nitroxides **5c**, **5d**, and **5e**. The formation of the last two nitroxides is easy to explain, as intermolecular hydrogen abstraction by photoexcited **5** from a methylene moiety of an ethyl group leads to a secondary-alkyl and a tertiary-alkyl radical; both can couple with NO to give two adventitious nitroso derivatives that can trap another secondary- or tertiary-alkyl radical to give **5d** and **5e**.

As for **5c**, while its spectral parameters leave little doubt about its identity, it is more difficult to rationalize its formation: although this may be thought to involve the secondary-alkyl nitroso derivative, the presence of the hydrogen atom directly bound to the nitroxidic nitrogen is difficult to account for.

2.6. *N*-Methylpropionamide, 6

As could have been foreseen, the photoreaction of **6** with MNP paralleled that of **2** leading to the detection of the two nitroxides **6a** and **6b** resulting from intermolecular hydrogen abstraction along

with that of the acyl-nitroxide **6c** originating from the trapping of a propionyl radical (β-scission) by MNP.

Likewise **2**, which upon photolysis in the presence of NO only led to the detection of nitroxide **2e**, **6** only afforded the symmetric nitroxide **6d** where two identical primary-alkyl fragments are bound to the nitroxidic function.

2.7. *N,N*-Dimethylpropionamide, 7

Four different nitroxides could be characterized when photolyzing **7** in the presence of MNP. Indeed, due to the intrinsic magnetic non equivalence of the two amidic methyl groups, hydrogen abstraction led to the two isomeric *tert*-butyl primary-alkyl nitroxides **7a** and **7b** along with the *tert*-butyl *tert*-alkyl nitroxide **7c**. As it had been the case for **6**, the *tert*-butyl propionyl nitroxide **6c** was also observed, the β-scission process from which it was originated being inhibited by the addition of some PILA 124.

In contrast with what observed with the structurally related amide **3**, both the two nitroxides detected when photolyzing **7** in the presence of NO reflected a β-scission process. Thus, the formation of the propionyl nitroxide **7d** may be thought to proceed as proposed in Scheme 4 (see Discussion) for the formation of **3c** and **5c**, while in principle that of **7e** may involve either trapping of a propionyl radical by $EtC(O)NMeCH_2NO$ (coupling of NO with the primary-alkyl radical from hydrogen abstraction) or that of the primary radical $EtC(O)NMeCH_2^{\bullet}$ by EtC(O)NO (coupling of NO with the propionyl radical from β-scission).

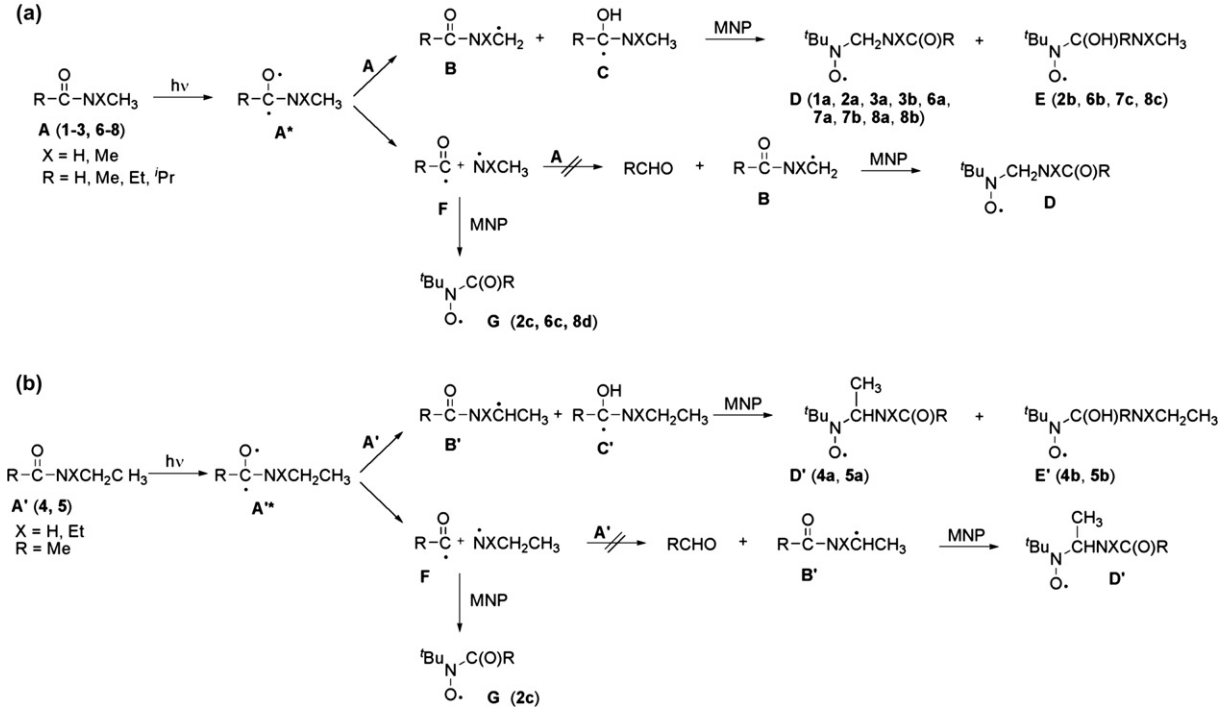
2.8. *N,N*-Dimethylisobutyramide, 8

Both intermolecular hydrogen abstraction and β-scission were evident in the photoreaction of **8** with MNP that led to the detection of five different nitroxides. Also in this case, due to the intrinsic non equivalence of the amidic methyl groups, hydrogen abstraction resulted in two isomeric primary-alkyl radicals that eventually led to nitroxides **8a** and **8b** along with the *tert*-butyl tertiary-alkyl nitroxide **8c**. β-Scission led instead to acyl nitroxide **8d**, while *tert*-butyl isopropyl nitroxide (**8e**) reflects decarbonylation of some isobutyroyl radicals prior to trapping by MNP.

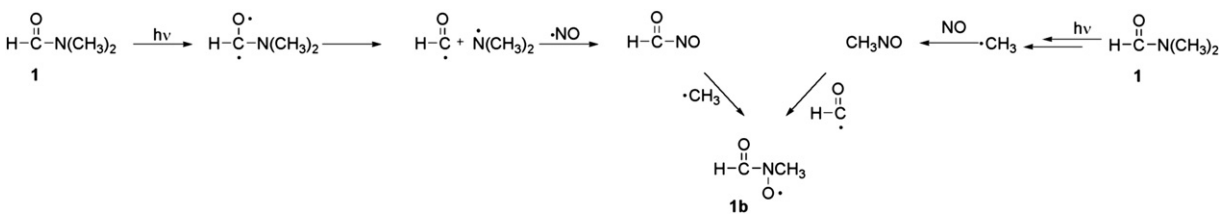
The detection of **8g** when **8** was photolyzed in the presence of NO reflects intermolecular hydrogen abstraction, while that of nitroxide **8f** requires the occurrence of both intermolecular hydrogen abstraction and β-scission.

3. Discussion

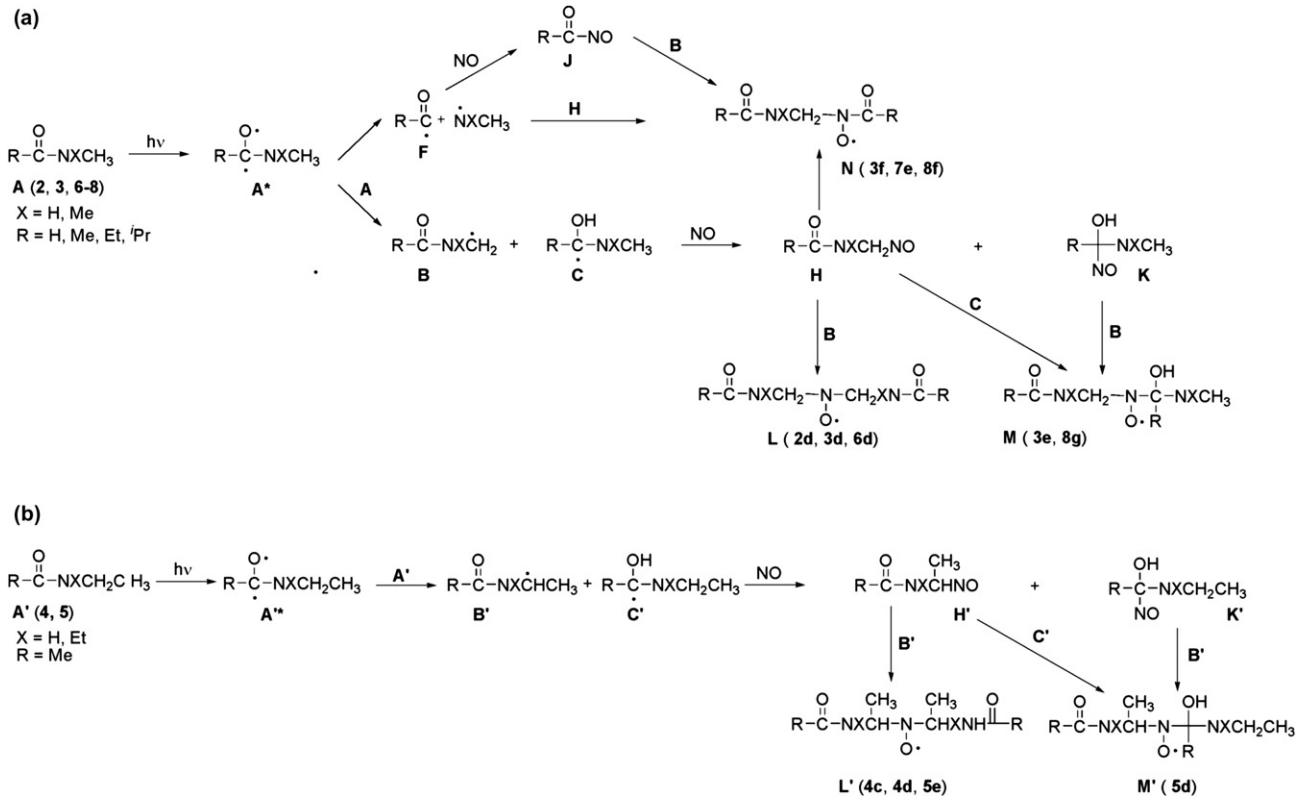
As the action of the UVA and UVB components of solar radiation on carbonyl compounds, and in particular amides, that are common components of skin proteins as well as therapeutic or cosmetic salves, balms, and soothing ointments used as skin sunscreens, and normally results in the onset of radical processes, we were prompted to investigate the photo behavior of some alkyl amides in the presence of NO, a reactive species naturally occurring in the human body.



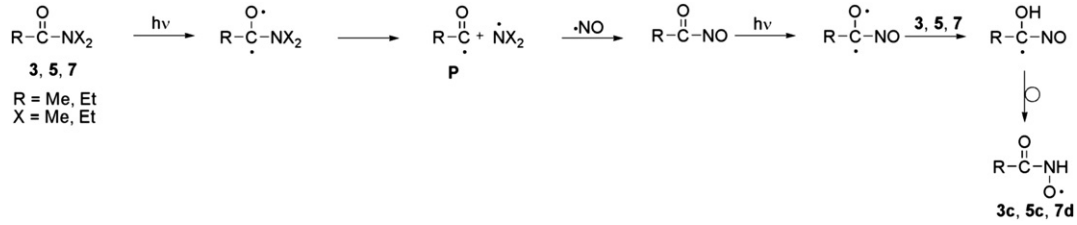
Scheme 1.



Scheme 2.



Scheme 3.



Scheme 4.

The photochemical behavior of alkyl amides has long since been the subject of investigation, and the initial proposal[15] of a type II photodecomposition of these substances with formation of alkenes and lower amides has been later reassessed[16,17] in favor of a type I photodecomposition involving acyl, alkyl, and aminyl radicals in the case of *N*,*N*-dialkyl amides, whereas unsubstituted alkyl amides were found to be stable to photolysis. Subsequent spin trapping studies carried out either in the presence or in the absence of H_2O_2 confirmed these results, and also led to the indirect detection of radicals resulting from hydrogen abstraction from the amides by photogenerated hydroxyl radicals.[18,19]

The occurrence of a type I process is confirmed also in the present case based on the detection of *tert*-butyl acetyl nitroxide **2c** in the photoreaction of **2**, **3**, **4**, and **5**, of *tert*-butyl propionyl nitroxide **6c** in the photoreaction of **6** and **7**, and of *tert*-butyl isobutyroyl nitroxide **8d** in the photoreaction of **8**. The corresponding acyl nitroxides were instead not detected upon photolysis of **1** in the presence of either MNP or NO.

The detection of nitroxides **1a**, **2a**,**b**, **3a**,**b**, **4a**,**b**, **5a**,**b**, **6a**,**b**, **7a**,**c**, and **8a**–**c** upon photolysis of amides **1**–**8** in the presence of MNP as a spin trap indicates that in all cases hydrogen abstraction from an *N*-alkyl group takes place. This must of course be an intermolecular process whereby the photoexcited (triplet) carbonyl abstracts a hydrogen atom from the alkyl group of a nearby amide molecule. This process is outlined in Scheme 1a for amides **1**–**3** and **6**–**8**.

The detection of nitroxides **E** and of acyl nitroxides **G** provides evidence that radicals **B** are formed via hydrogen abstraction by the triplet of the photoexcited amide rather than by the acyl radical formed in the β-scission of **A***. For *N*-ethyl- and *N*,*N*-diethyl-acetamide **4** and **5**, respectively, hydrogen abstraction takes place from one of the methylenic groups (see Scheme 1b).

Photolysis of amides **1**–**8** in the presence of NO also led in all cases to the EPR detection of nitroxides. Because of the replacement of MNP with NO, on the other hand, these nitroxides differed from those detected with MNP despite the fact that the radicals involved in their formation were the same, reflecting the occurrence of the same processes, i.e., intermolecular hydrogen abstraction and β-scission. Indeed, being a radical, NO acts as a radical scavenger and reacts very quickly, coupling with the radicals formed in the photolysis of **1**–**8** giving rise to adventitious alkyl- or acyl-nitroso compounds, which in turn behave as spin traps for other radicals being generated.

Having been reported that photolysis of DMF **1**, besides giving formyl and dimethylaminyl radicals (type I β-scission), may also afford methyl radicals,[13] the two converging routes outlined in

Scheme 2 can be envisaged to account for the detection of acyl nitroxide **1b**.

Compound **1b** was in fact the only detected radical, and we could not gather any evidence of the formation of *N*-nitroso-amines nor of the trapping of aminyl radicals. Incidentally, this failure to intercept alkylaminyls applies to all the substrates of the present study.

Nitroxide **2d**, the only radical detected upon photolysis of **2** in the presence of NO, is unmistakable evidence of intermolecular hydrogen abstraction whereby a photoexcited molecule **2*** abstracts a hydrogen from an *N*-methyl group of a nearby molecule giving a primary-alkylamino radical, which couples with NO. The resulting nitroso compound traps another primary-alkylamino radical leading to the symmetric nitroxide **2d** (see Scheme 3a and b). The same mechanism can be applied to the photoreaction of the other mono- or di-*N*-alkyl amides, in particular to account for the formation of **3d** from **3**, **4c** and **4d** from **4**, **5e** from **5**, and **6d** from **6**. As hydrogen abstraction simultaneously generates two-carbon centered radicals, the formation of mixed nitroxides is also possible: this is the case in the formation of **3e** from **3**, **5d** from **5**, and **8g** from **8**. The detection of two symmetric nitroxides in the photolysis of **4** deserves a special comment. Indeed, the spectral parameters of **4c** and **4d** suggest that they are diastereoisomers with the methine hydrogen atoms lying closer the C–N–O plane in the case of **4d** than in the case of **4e**. No such isomerism was exhibited by the structurally related nitroxide **5e**, possibly because the ethyl group replacing the NH hydrogen destabilizes the conformation where the methine hydrogen atoms lie close to the C–N–O plane.

Two kinds of acyl nitroxides were also identified among the radicals detected when photolyzing amides **1**–**8** in the presence of NO. Compounds **3f**, **7e**, and **8f** are alkyl acyl nitroxides whereas **3c** and **7d** are simple acyl nitroxides. The formation of the three former radicals can be readily accounted for as outlined in Scheme 3a by admitting the involvement of acyl radicals originated via β-scission (**F**) and carbon centered radicals from hydrogen abstraction (**B**).

In principle the nitroxides may be formed in any of the two ways indicated in Scheme 3a, although we favor that involving the alkylnitroso instead of the acyl nitroso derivatives, the former being the more stable species.

The formation of **3c** and **7d** must also involve β-scission of photoexcited **3*** or **7*** to give the appropriate acyl radicals **P**, the coupling of which with NO would lead to acyl nitroso derivatives, an overall possible mechanism being exemplified in Scheme 4.

Although we only consider the last steps of the route to **3c** and **7d** shown in this scheme to be hypothetic al, we wish to note that the detection of acyl nitroxides upon photolysis of acyl nitroso derivatives has actually been reported.[20]

With radical **5c**, its formation must involve intermolecular hydrogen abstraction from the methylenic group of **5** by a second photoexcited **5*** molecule with subsequent formation of a secondary-alkyl nitroso derivative by coupling with NO (see **H′**). Although nitroso compounds are normally inefficient hydrogen abstractors, it may be tentatively hypothesized that **5c** is eventually formed through a route formally akin to the last two steps of Scheme 4.

Finally, we wish to note that the present results reassess what was wrongly reported in a paper on the EPR characterization of the nitroxides formed in the photoreactions of some amides with NO.[21] Although there seems to be a reasonable consistency between the spectral parameters we determined for **3a**, **3b**, **6a**, **6b**, **7a**, **7b**, **8a**, **8b** and those reported by these authors for some structurally related nitroxides, we cannot avoid noting that many of the species reported in that paper have been clearly mis-identified. In particular this is the case of nitroxide $CH_3CH(OH)N(O^•)C(O)CH_3$ that in fact is the well documented $HN(O^•)C(O)CH_3$,[22] of the alleged, and in our knowledge still elusive diaminonitroxide $(CH_3)_2NN(O^•)N(CH_3)_2$, identified on the basis of an EPR spectrum that was actually simply due to dimethylaminoxyl radical itself,[23,24] and of diacyl nitroxides $RC(O)N(O^•)C(O)R$ for which spectral parameters are given that are instead in line with acyl *tert*-alkyl nitroxides $RC(O)N(O^•)R'$.[25] A more detailed discussion on the erroneous identification of these radicals is available in Supplementary data.

It may be worth re-emphasizing that NO, being a paramagnetic species, acts as a scavenger towards the radicals produced in the photoreactions of amides **1**–**8**, affording diamagnetic nitroso compounds that in turn can act as spin traps towards other radicals. In other words, each unit of NO reacts directly or indirectly with two radical units, and the formation of the observed nitroxides may be seen as a process akin to the one taking place when nitric oxide is used to double-trap biradicals leading to cyclic nitroxides.[26,27]

Sunlight induced skin reactions are well known and are commonly targeted by biological end points such as erythema, persistent pigment darkening or immunosuppression. These processes are claimed to involve free radicals, which can be formed at all wavelengths and in different skin layers, from the horny layer up to a depth of 30 mm in the case of near-IR irradiation.[2]

The EPR spin-trapping technique allows the study and detection of these species, even if the effect of the spin traps on the cells and their potential toxicity must be taken into account. However, the use of spin traps for direct measurements in biological systems has been limited due to the poor stability of the resulting adducts in viable systems where the aminoxyls are readily converted to EPR silent products.[28]

Since it is reported that NO participates in the regulation of skin functions such as circulation, UV-mediated melanogenesis,[25] sunburn erythema, and the maintenance of the protective barrier against microorganisms we thought it interesting to investigate whether it could also exert a protective-role acting as spin trap towards radicals induced by UV radiations.

Actually, not much has been reported on the excitation mechanisms triggered in the skin by radiations, on the formation of radical species, and on the molecules that may be involved in the initial photoexcitation process. Since the outermost layer of the epidermis, the stratum corneum (SC), consists of dead, flattened cells embedded in lipid lamellar regions where a series of ceramides is present, which are characterized by the presence of amido groups in their structure, these species could be involved in the initial photoexcitation process.[29] Peptides, critical component of the innate immune response in the skin as antimicrobial,[30] or species such as nicotinamide (vitamin B3)[31] are also present in the epidermis. In all these species the amide functions are present, and it is sensible hypothesizing their involvement in photo-activation processes. Moreover, in skin epidermis, NO is also produced by keratinocytes in response to UV radiation.[32]

In this light, the study of the photolysis of alkyl amides in the presence of NO, that could act as endogenous-type spin trap, could provide valuable information mimicking in vivo conditions.

In conclusion, in what we are aware to be a very optimistic scenario, the use of NO as an 'indirect' spin trap might appear potentially useful for studies in living cells, making unnecessary the preliminary stability and toxicity trials to determine the appropriateness of exogenous spin traps.

4. Experimental

4.1. Chemicals

2-Methyl-2-nitrosopropane (MNP), amides **1**–**8**, 2-ethoxythioxanthone (PILA 124), and all other reagents were purchased

from Aldrich in the highest purity grade commercially available, and were used as received. NO gas, 99%, was supplied by Matheson.

4.2. Apparatus

All EPR spectra were recorded on an upgraded X-band Bruker ER200/ESP300 spectrometer equipped with a NMR gaussmeter for field calibration and a frequency counter for the determination of *g*-factors that were corrected with respect to that of perylene radical cation in concentrated sulfuric acid (g=2.0025$_8$).

A custom-made Suprasil® quartz flat flow cell (3×6×0.15 mm) was used. The flow of the solution was ensured by a motor-driven syringe and could be varied as appropriated. The cell was irradiated with the light from a 250 W high pressure Hamamatsu Hg-lamp focalized via an optical fiber light guide into the center of the spectrometer cavity.

The temperature was controlled through a standard variable temperature set up and monitored with a Chromel-Alumel thermocouple inserted inside the sample tube.

The EPR spectra were computer simulated using a self minimizing software based on a Monte Carlo procedure.[33]

4.3. Photolysis in the presence of MNP

Using a porous-bottom flask, acetonitrile (10 ml) was deoxygenated by bubbling pure N_2 gas for circa 30 min, before adding the alkyl amide (final concentration 2 M). After nitrogen-purging the solution for 10 more minutes, MNP was added (10^{-3} M) and the EPR experiment immediately run. It was carried out by photolyzing the solution continuously flowing through a flat cell placed inside the EPR spectrometer cavity.

Additional experiments were also accomplished adding to the final solution a substantial amount of the photoinitiator PILA 124 and filtering the incident UV radiation through a 400 nm long-pass filter in order to avoid light absorption by the amides.

4.4. Photolysis in the presence of NO

Using a porous-bottom flask, acetonitrile (10 ml) was deoxygenated by bubbling N_2 gas, for circa 30 min; the solvent was then purged with NO for 20 more minutes, the resulting final NO concentration being circa 10^{-3} M. To avoid pollution by adventitious NO_x, such as NO_2, the NO stream was first passed through a concentrated NaOH aqueous solution to trap the undesired species. Finally, the amide was added (final concentration 2 M) a few minutes before the end of the NO purging. The solution, continuously flowing through the flat cell, was then photolysed inside the cavity of the EPR spectrometer.

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Supplementary data

All relevant EPR spectra and simulations along with a critical discussion of the misassignments reported in Ref. 21 are available as Supplementary data. Supplementary data related to this article can be found online at doi:10.1016/j.tet.2012.01.066.

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