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Laboratory note

Synthesis, characterization, crystallographic analysis, antifungal and genotoxic properties of some 1-methyl-1*H*-imidazoles

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Summary — A number of 1-methyl-1*H*-imidazole derivatives and some of their oxygenated products were synthesized. An HPTLC technique for following the oxidation reactions in the different experimental conditions used was applied. The X-ray crystal structures of 1-methyl-2-methylsulfanyl-5-nitro-1*H*-imidazole, 2-methanesulfinyl-1-methyl-5-nitro-1*H*-imidazole and 2-methanesulfonyl-1-methyl-5-nitro-1*H*-imidazole were determined. The compounds obtained were investigated for antimycotic and genotoxic activities. The compounds tested were found to exert very low growth inhibition against yeasts and moulds. Moderate antifungal properties against dermatophytes were demonstrated for 5-nitro derivatives. 2-Methanesulfonyl-1-methyl-5-nitro-1*H*-imidazole was the most active substance. All 5-nitroimidazoles were genotoxic in *Bacillus subtilis rec*-assay, *Salmonella* microsome test and in *Saccharomyces cerevisiae* mitotic segregation assay. Structure–activity relationships are discussed.

1-methyl-1*H*-imidazole derivative/ HPTLC / X-ray structure/ antifungal activity/ genotoxicity

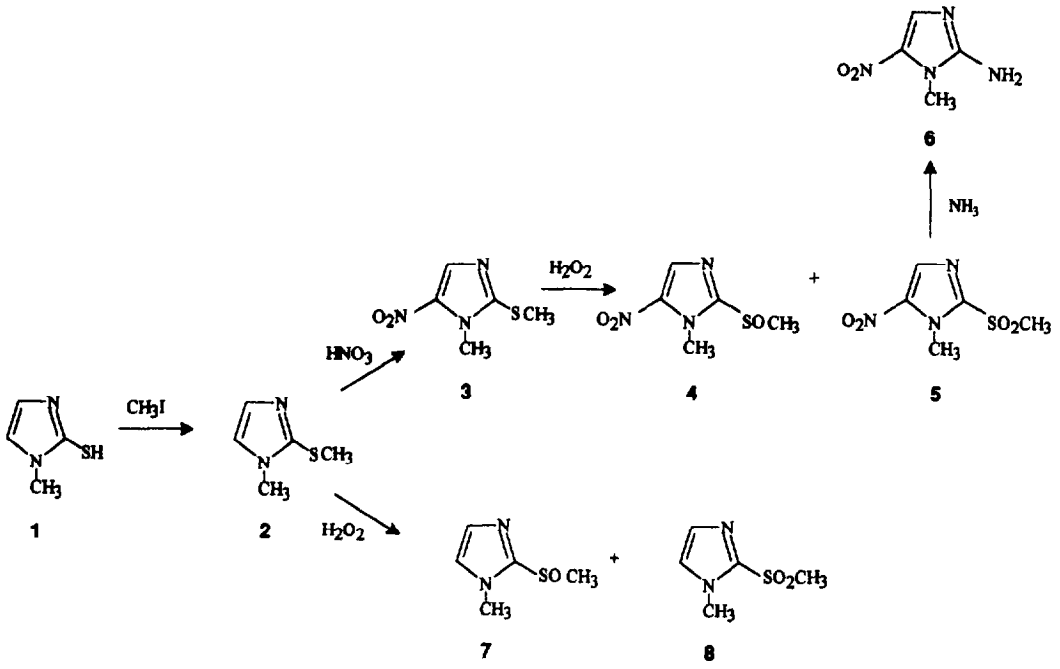
Introduction

Nitrogen heterocycles are extremely important fungicides [1]. Of these, imidazole derivatives have been extensively studied since the discovery of the broad spectrum antimycotic activity of bifonazole, clotrimazole, econazole, isoconazole, ketoconazole, miconazole and tioconazole, which are at present very important chemotherapeutic agents. These molecules act by inhibiting several cytochrome P-450-dependent enzymes involved in the biosynthesis of ergosterol which has an important function in fungal membrane stability [1]. Moreover, several 5-nitroimidazoles, such as metronidazole, dimetridazole, ornidazole and tinidazole, have been introduced in the therapy of amoebic, trichomonal, giardial and anaerobic bacterial infections. Damage to DNA is the essential mechanism of the antimicrobial activity of nitroimidazoles [2] since binding to DNA upon the reduced drug causes strand breakage.

By changing the chemical substituents at the 1- and 2-positions of the 5-nitroimidazole, it is possible to obtain molecules that are active against a broader range of microorganisms [3]. In view of this observation, we synthesized 1-methyl-2-methylsulfanyl-5-nitro-1*H*-imidazole **3** by nitration of 1-methyl-2-methylsulfanyl-1*H*-imidazole **2**; 2-methanesulfinyl-1-methyl-5-nitro-1*H*-imidazole **4** and 2-methanesulfonyl-1-methyl-5-nitro-1*H*-imidazole **5** were prepared by oxidation of **3** (scheme 1); and 1-methyl-5-nitro-1*H*-imidazol-2-ylamine **6** was obtained by reaction of **5** with ammonia. The oxidation reaction was monitored by means of an HPTLC technique to establish the best experimental conditions. These compounds were characterized by means of UV, IR, and ^{1}H-NMR spectroscopy, and the crystallographic parameters of **3**, **4** and **5** were determined by X-ray analyses. These 1-methyl-5-nitroimidazole derivatives are structurally related to metronidazole and dimetridazole and were tested for antifungal activity.

The present study also examines the genotoxicity of the imidazoles described since compounds **3–6** contain 'alerting' nitro and amino groups [4, 5]. In fact several 5-nitroimidazoles have been shown to be mutagenic in *Salmonella typhimurium* strains [6].

For comparative purposes we also investigated the biological properties of the parent imidazoles and structurally related derivatives without a 5-nitro substituent (scheme 1: 1-methyl-1*H*-imidazole-2-thiol **1**, 1-methyl-2-methylsulfanyl-1*H*-imidazole **2**, 2-methanesulfinyl-1-methyl-1*H*-imidazole **7**, 2-methanesulfonyl-1-methyl-1*H*-imidazole **8**). These compounds have been synthesized recently and were described in a previous paper [7].



Scheme 1.

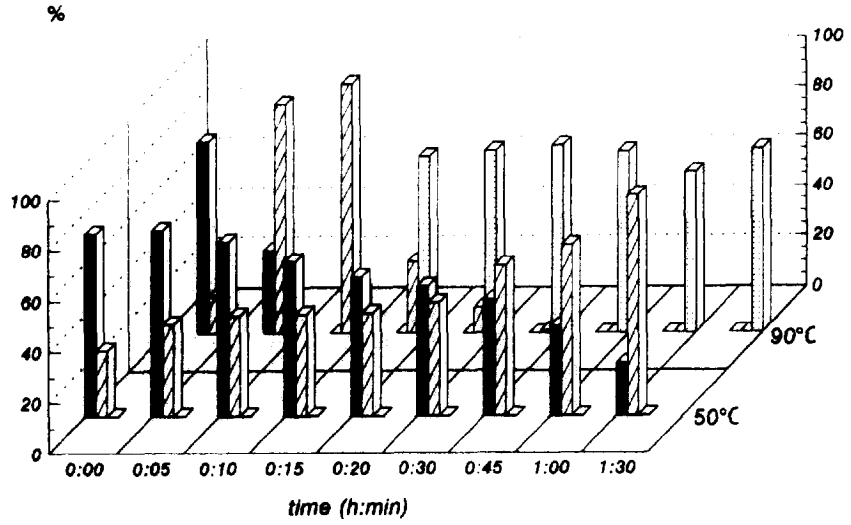


Fig 1. Oxidation of 1-methyl-2-methylsulfanyl-5-nitro-1*H*-imidazole **3** hydrogen peroxide with 2 equiv, 50 and 90°C. ■ **3**; ▨ **4**; ▩ **5**.

Results and discussion

Chemistry

Compounds **1–8** were obtained according to scheme 1. Considering that the oxidations of **2** to **7** and **8**, and **3** to **4** and **5** strictly depend on the experimental conditions, an HPTLC technique was set up to monitor this reaction at 50 and 90°C, and with 2 or 4 equiv of hydrogen peroxide. Figures 1 and 2 show the course of the oxidation of **3** to **4** and **5**. It can be seen that a) at 50°C compound **4** forms immediately, while **5**

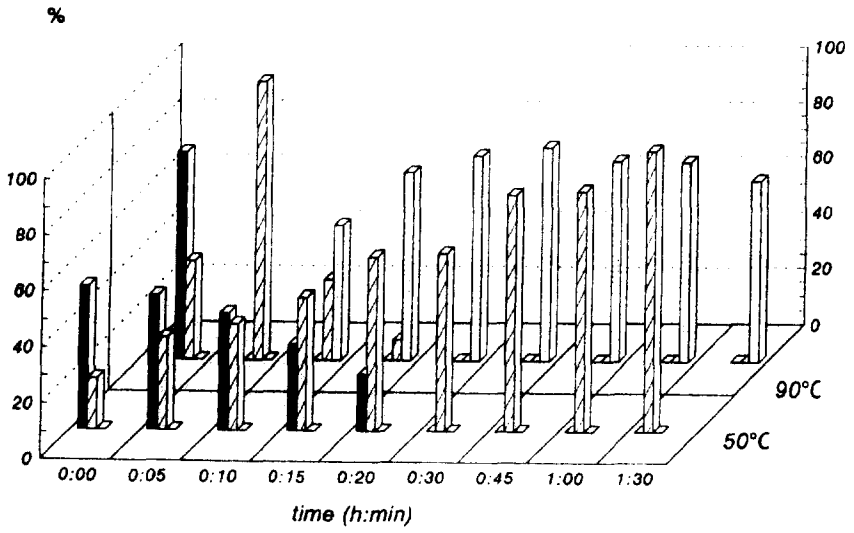


Fig 2. Oxidation of 1-methyl-2-methylsulfanyl-5-nitro-1*H*-imidazole **3** hydrogen peroxide with 4 equiv, 50 and 90°C. ■ **3**; ▨ **4**; ▩ **5**.

does not appear at this temperature; with 4 equiv of hydrogen peroxide, **4** is produced in greatest yields; and b) at 90°C sulfoxide **4** appears early, and then **5** appears; at this temperature the concentration of oxidating agent does not influence the reaction.

The crystal structures of compounds **3** (fig 3) and **4** (fig 4) are built up of one independent molecule, whereas the asymmetric unit of compound **5** contains two crystallographically independent molecules (fig 5). The bond distances and bond angles involving non-hydrogen atoms are reported in table I, for all compounds.

The dimensions of the imidazole ring in compounds **3** and **4** and in the independent molecules of **5**, do not differ significantly and agree well with those reported for other nitro-substituted imidazoles [8–12]. Comparison with corresponding vectors in the unsubstituted imidazole [13] confirms that the C(2)-N(3) bond is the shortest, indicating a more localized double bond between these atoms as a common feature of the chemistry of the nitroimidazoles [8–12]. The present results also confirm the previously observed opening of the angle at the atom to which the nitro group is bonded, and the closing of the two adjacent ring angles, probably owing to the electron-withdrawing property of the substituent [8]. Likewise, the $-SCH_3$, $-SOCH_3$, or $-SO_2CH_3$ substituents at the C(2) carbon give rise to significant widening of our N(1)-C(2)-N(3) angles, which range from 113.5(3) to 114.1(3)°, and should be compared with a value of 111.9(1)° of the unsubstituted imidazole [13].

The bond distances within the imidazole ring are apparently unaffected by the presence of the above-cited substituents at the C(2) atom. Their dimensions, as well as those of the nitro groups, are quite normal [14].

As a further common feature of imidazole derivatives, in the present cases the five ring atoms are strictly coplanar, the maximum deviations from mean plane being ±0.002 Å in **3**, ±0.004 Å in **4**, and ±0.006 and ±0.003 Å for the two independent molecules of **5**.

The molecular conformation of compound **3** is characterized by close coplanarity of all its non-hydrogen atoms; the maximum atomic deviation from the mean plane of the imidazole ring is 0.064 Å by the

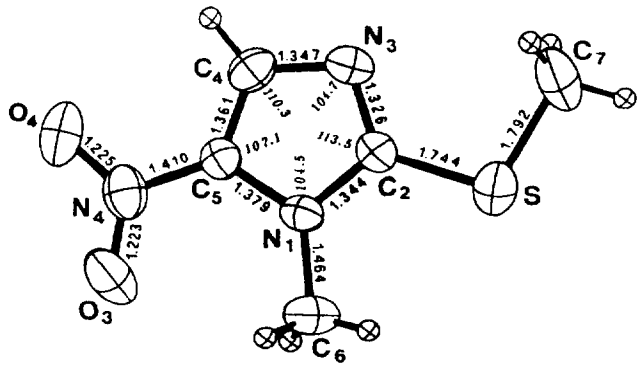


Fig 3. Ortep [24] drawing of the molecular structure of compound **3** showing atom numbering scheme, thermal motion ellipsoids (40%), bond distances (Å) and bond angles (degrees).

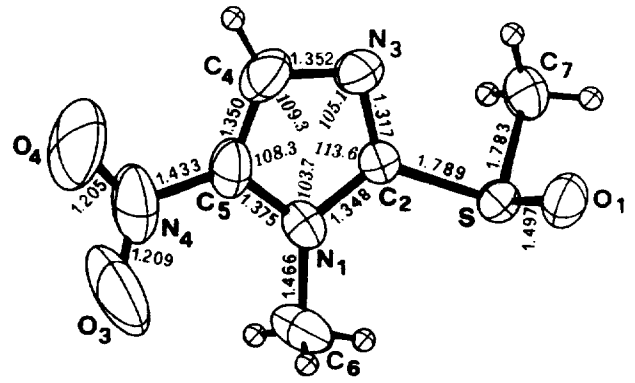


Fig 4. Atom numbering scheme, thermal ellipsoids (50%), bond distances (Å) and bond angles (degrees) for compound **4**.

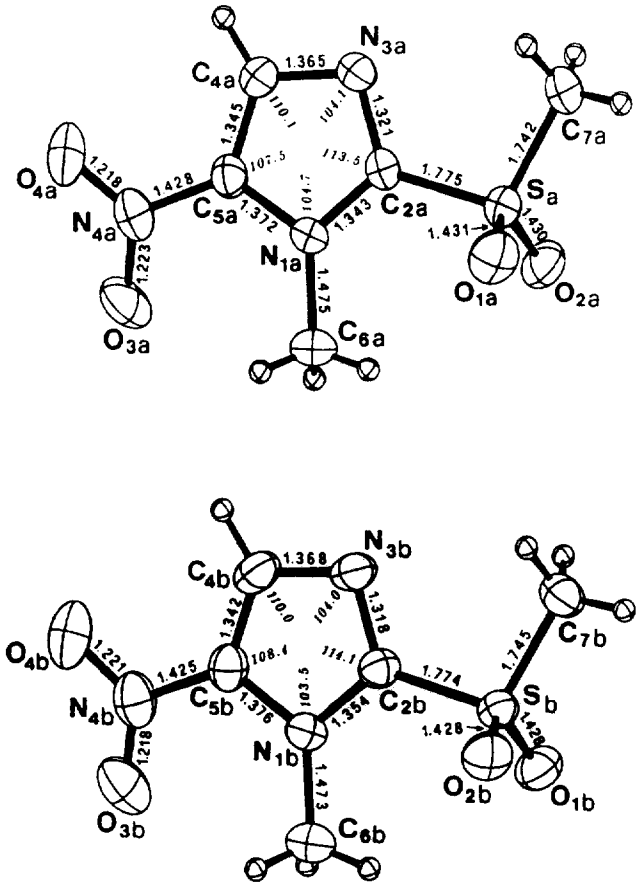


Fig 5. Atom labelling, thermal ellipsoids (40%), bond distances (Å) and bond angles (deg) for the two crystallographically independent molecules of compound **5**.

C(7) methyl carbon. The planar nitro group makes a dihedral angle of 1.1° with the imidazole ring plane. The molecular packing of compound **3** appears to be caused by aromatic ring-stacking interactions, typical of unsaturated cyclic compounds. The overlap of the planar molecules in parallel layers along the *a* cell axis is characterized by some short (~3.40 Å) interlayer van-der-Waals contacts.

The molecular conformation of compound **4** differs significantly from that of **3** in the orientation of the substituent at the C(2) atom. The N(3)-C(2)-S-C(7) torsion angle is 3.1(4)° in **3** and –39.4(2)° in **4**. As a consequence, the C(7) and O(1) atoms of compound **4** are strongly displaced, and at opposite sides (–0.926 and 1.520 Å, respectively) from the imidazole mean plane, whereas all other non-hydrogen atoms lie close to the ring plane. The nitro group is slightly twisted with respect to the imidazole ring, the dihedral angle between their mean planes is 3.9°.

The molecular packing of compound **4** is completely different from that of **3**, and is characterized by more (15 contacts less than 3.60 Å) and stronger (3 contacts less than 3.08 Å) van-der-Waals interactions. These interactions mainly involve the sulphinic oxygen, and could play a role in the orientation of the $-SOCH_3$ substituent.

There is very good agreement between corresponding bond distances and bond angles in the two independent molecules of compound **5**. The loss of crystallographic equivalence appears to be due to the conformational differences which involve the orientations of both the $-SO_2CH_3$ and the $-NO_2$ substituents, both differing by a rotation of about 11° around the bond to the imidazole ring. The N(3a)-C(2a)-S(a)-C(7a) torsion angle is 0.2(4)°, hence close to that of compound **3**, where the corresponding value for molecule b is –11.5(4)°. Likewise, the dihedral angles between mean planes through the imidazole ring and the nitro group are 21.5 and 10.5° for molecules a and b, respectively.

It is of interest to note that the first twist angle greatly exceeds the highest value (10.0°) that we have found in the literature [15]. As previously observed [12], this effect appears due to the steric environment of the nitro substituent. It may also be underlined that the C-N bond distances are short and consistent with an extended exocyclic conjugation group between the nitro groups and the imidazole rings, in spite of relevant twist angles. Another interesting feature of the structure of compound **5** is the crystal packing. The molecules are linked together by a surprisingly large number (37 contacts less than 3.60 Å) of strong van-der-Waals interactions. Most of the shortest intermolecular contacts (2.891–2.979 Å) involve sulphonic oxygens and nitrogen atoms of the nitro groups. As for compound **4**, these interactions appear to play a

Table I. Bond distances (Å) and bond angles (degrees) with estimated standard deviations in parentheses.

Bond	*3*	*4*	*5*	
			Molecule a	*Molecule b*
C(2)-N(1)	1.344(5)	1.348(2)	1.343(4)	1.354(4)
C(5)-N(1)	1.379(5)	1.375(5)	1.372(4)	1.376(4)
C(6)-N(1)	1.464(5)	1.466(3)	1.475(4)	1.473(4)
N(3)-C(2)	1.326(5)	1.317(2)	1.321(4)	1.318(4)
S-C(2)	1.744(4)	1.789(2)	1.775(3)	1.774(3)
C(4)-N(3)	1.347(5)	1.352(3)	1.365(4)	1.368(5)
C(5)-C(4)	1.361(6)	1.350(3)	1.345(5)	1.342(5)
N(4)-C(5)	1.410(6)	1.433(3)	1.428(4)	1.425(5)
C(7)-S	1.792(6)	1.783(2)	1.742(4)	1.745(4)
O(1)-S	–	1.497(2)	1.430(2)	1.428(3)
O(2)-S	–	–	1.431(3)	1.428(2)
O(3)-N(4)	1.223(5)	1.209(4)	1.223(4)	1.218(4)
O(4)-N(4)	1.225(5)	1.205(4)	1.218(4)	1.221(4)
C(5)-N(1)-C(2)	104.5(3)	103.7(2)	104.7(3)	103.5(3)
C(6)-N(1)-C(2)	125.4(4)	126.7(2)	126.6(3)	126.5(3)
C(6)-N(1)-C(5)	130.1(4)	129.6(2)	128.5(3)	129.8(3)
N(3)-C(2)-N(1)	113.5(3)	113.6(2)	113.5(3)	114.1(3)
S-C(2)-N(1)	121.4(3)	121.9(1)	123.1(2)	124.0(3)
S-C(2)-N(3)	125.1(3)	124.4(1)	123.4(2)	121.8(3)
C(4)-N(3)-C(2')	104.7(3)	105.1(2)	104.1(3)	104.0(3)
C(5)-C(4)-N(3)	110.3(4)	109.3(2)	110.1(3)	110.0(3)
C(4)-C(5)-N(1)	107.1(4)	108.3(2)	107.5(3)	108.4(3)
N(4)-C(5)-N(1)	125.6(4)	124.0(2)	124.8(3)	124.6(3)
N(4)-C(5)-C(4)	127.3(4)	127.6(2)	127.7(3)	127.0(3)
C(7)-S-C(2)	99.9(2)	96.6(1)	102.8(2)	102.8(2)
O(1)-S-C(2)	–	105.7(1)	107.4(2)	107.8(2)
O(2)-S-C(2)	–	–	106.9(2)	107.4(2)
O(2)-S-O(1)	–	–	117.8(2)	117.7(2)
C(7)-S-O(1)	–	107.1(1)	110.1(2)	109.5(2)
C(7)-S-O(2)	–	–	110.6(2)	110.4(2)
O(3)-N(4)-C(5)	118.6(4)	119.4(3)	118.5(3)	119.3(4)
O(4)-N(4)-C(5)	117.4(4)	116.2(3)	116.2(3)	115.7(4)
O(4)-N(4)-O(3)	124.0(4)	124.3(3)	125.2(3)	124.9(4)

determining role in the orientation of the substituent with respect to the imidazole ring.

Antifungal activity

The *in vitro* antifungal activity of compounds **1–8** was determined on yeasts and moulds of medical interest by the two-fold dilution technique [16]. The results are reported in table II. The imidazoles tested generally displayed low activity: the MIC values were higher than 200 μg/ml for yeasts and 100–200 μg/ml for moulds. Only compound **3** showed a limited antifungal activity against most of the yeasts tested (MIC = 100–200 μg/ml), whereas the effectiveness of compounds **5** and **6** is restricted to *Cryptococcus neoformans*. Against filamentous fungi an inhibitory activity was shown by compounds **3–6**; **5** was the most active substance. Dermatophytes were particularly sensitive. *Epidermophyton floccosum* exhibited a more marked susceptibility. *Candida parapsilosis*,

Table II. Antifungal activity (MIC in μg/ml) of compounds **1–8**.

Microorganism	*Compound*								
	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*MICO*
Yeasts									
S cerevisiae ATCC 9763	> 200	> 200	100	> 200	> 200	> 200	> 200	> 200	12
C albicans	> 200	> 200	100	> 200	> 200	> 200	> 200	> 200	6
C guilliermondii	> 200	> 200	200	> 200	> 200	> 200	> 200	> 200	0.3
C parapsilosis	> 200	> 200	> 200	> 200	> 200	> 200	> 200	> 200	0.7
C tropicalis ATCC 1369	> 200	> 200	200	> 200	>200	> 200	> 200	> 200	6
C neoformans	> 200	> 200	200	> 200	100	200	> 200	> 200	0.3
Moulds									
A flavus	> 50	> 100	> 100	> 100	> 100	> 100	> 100	> 100	50
A fumigatus	> 50	> 100	> 100	100	50	> 100	> 100	> 100	10
A niger ATCC 6275	> 200	> 200	200	> 200	100	> 200	> 200	> 200	3
E floccosum	> 50	> 100	100	1	0.1	100	> 100	> 100	0.01
M gypseum	> 50	> 100	> 100	100	50	> 100	> 100	> 100	10
P boydii	> 50	> 100	> 100	> 100	> 100	> 100	> 100	> 100	100
S schenckii	> 50	> 100	> 100	> 100	> 100	> 100	> 100	> 100	50
T interdigitalis	> 50	> 100	> 100	> 100	50	> 100	> 100	> 100	1
T mentagrophytes	> 50	> 100	> 100	> 100	50	> 100	> 100	> 100	1
T rubrum	> 50	> 100	100	50	10	> 100	> 100	> 100	1
T soudanense	> 50	> 100	100	50	5	100	> 100	> 100	0.1
T violaceum	> 50	> 100	50	50	5	100	> 100	> 100	0.1

The results are reported as arithmetic averages of MICs. Miconazole (MICO) is used as a reference compound.

Table III. DNA-damaging activity in the *B subtilis rec*-assay evaluated by the plate diffusion procedure and the two-fold dilution method.

Compound	*Plate diffusion method*				*Dilution method*		
	Amount (μg/disk)	*Inhibition halo (mm)*		*Activity*	*MIC (μg/ml)*		*Activity*
		rec$^+$	*rec*$^-$	*rec*$^-$/*rec*$^+$	*rec*$^+$	*rec*$^-$	*rec*$^+$/*rec*$^-$
1	1200	9	9	1	> 1600	> 1600	–
2	1200	9	9	1	> 1600	> 1600	–
3	1000	32	48	**1.5**	25	1.5	**16.7**
4	1000	28	36	**1.3**	50	3	**16.7**
5	1000	26	33	**1.3**	25	3	**8.3**
6	1000	10	16	**1.6**	1600	200	**8**
7	1200	9	9	1	> 1600	> 1600	–
8	1200	9	9	1	> 1600	> 1600	–
Controls[a]							
AMP	20	35	35	1	0.03	0.03	1
CAF	30	20	20	1	5	5	1
RIF					0.1	0.1	1
MMS	300	10	31	**3.1**	400	25	**16**
MIT C					0.15	0.015	**10**

Positive results are indicated in bold. [a]AMP, ampicillin; CAF, chloramphenicol; RIF, rifampicin; MMS, methyl methanesulfonate; MIT C, mitomicin C.

Aspergillus flavus, *Pseudallescheria boydii* and *Sporothrix schenckii* turned out to be completely resistant.

It is interesting to note that, of the imidazoles tested, only compounds carrying the nitro group showed a certain inhibitory activity against yeasts and moulds. In fact the nitro substitution present in compounds **3**, **4** and **5** increased the activity of the corresponding compounds **2**, **7** and **8**. The replacement of the mercapto group at position 2 by an amino group (compound **6**) did not enhance the fungitoxicity of this series of 5-nitroimidazoles.

Genotoxicity

Compounds **1–8** were tested *in vitro* for genotoxicity in the *Bacillus subtilis rec*-assay, *Salmonella*-microsome test and *Saccharomyces cerevisiae* mitotic segregation assay. The use of these systems with different endpoints allows us to identify and partially characterize the type of damage caused by a chemical. The *rec*-assay test demonstrates the ability of the compounds to cause damage to bacterial DNA [17]. The chemically induced mutations in prokaryotes and lower eukaryotes were studied by the *Salmonella*-microsome test (reverse mutation assay, Ames test) in *S typhimurium* [18] and the mitotic segregation test in *S cerevisiae* [19], respectively.

As regards the *B subtilis rec*-assay, all compounds were tested by two different procedures (table III). In the plate diffusion method, compounds **1**, **2**, **7** and **8** did not inhibit the growth of either of the bacterial strains. However all nitroimidazoles producing a larger zone of growth inhibition against the repair-deficient strain as compared to the repair-proficient strain were effective DNA-damaging agents.

To improve *rec*-assay sensitivity, a preincubation procedure was also applied (data not shown). However, in this case no inhibition halo was observed for compounds **1**, **2**, **7** or **8**.

Because the absence of inhibition against the tester strains may be due to the poor solubility and diffusion of the compounds in the agar, we also tested DNA-damaging activity in liquid medium (two-fold dilution method). MIC data reported in table III confirm the stronger activity of all 5-nitroimidazoles against the recombination repair-deficient strain. The other imidazoles tested exhibited no difference in inhibition of cellular growth between repair deficient and wild bacteria.

In the evaluation of the mutagenic potential by *Salmonella*-microsome assay, different concentrations of the substances were tested. The results are presented in table IV: for negative compounds the higher non-toxic dose tested is reported, whereas for active compounds the dose producing the highest mutagenic response in the linear portion of the dose–response curve is reported. Compounds **1**, **2**, **7** and **8** displayed no mutagenic activity up to 2000 μg/plate, but all 5-nitro-substituted imidazoles **3–6** were mutagenic for the *S typhimurium* TA 100 strain, both in the absence and in the presence of S9-mix. This strain showed the highest sensitivity to the products. In addition, compound **6** exhibited mutagenicity for TA 1535, and all 5-nitroimidazoles, in the absence of metabolic activation, enhance the number of TA 98 revertant colonies, which, for compound **5**, is almost triple. This suggests that all active compounds may induce both base-pair and frame-shift-type mutations. In all cases the mutagenicity of nitro-substituted imidazoles is reduced following metabolic activation, which suggests that they act as direct-acting mutagens. Compound **6**, which carries an aromatic amino group 'alerting' for mutagenicity with microsomal activation, also exhibits a higher mutagenicity in the absence of S9-mix. For positive compounds the mutagenic activity, expressed as TA 100 revertants/μmol in the absence of metabolic activation, was calculated from data obtained in the dose-dependent concentration range. Derivative **3** was found to be the most mutagenic compound, its potency being 1.1×10^4 revertants/μmol, whereas those of compounds **4**, **5** and **6** were 8.9×10^3, 8.8×10^3 and 4.2×10^2 revertants/μmol.

Structure–activity relationships reveal that the mutagenicity of 1-methyl-2-methylsulfanyl-5-nitro-1*H*-imidazole is higher than that of oxidized derivatives **4** and **5**, which are comparable in potency. It was noted that the replacement of the mercapto groups by an amino group in the 2-position of the imidazole molecule leads to a decrease in mutagenic potency.

All the compounds were also tested by the preincubation procedure, a useful method that can detect weak mutagens. Even in these conditions, no mutagenicity can be detected for compounds **1**, **2**, **7** and **8** (data not shown). However, preincubation enhances the activity of all 5-nitroderivatives.

The genotoxicity of compounds **3–6** was also demonstrated in the *S cerevisiae* 6117 test in the absence of metabolic activation. 5-Nitroimidazoles stimulate the frequency of mitotic convertants. On the other hand, no increase in the frequency of mitotic recombinants was detected. As regards other imidazoles, negative results were found in both mitotic cross-over and gene conversion. Table V presents data concerning spontaneous segregation and segregation induced by mutagenic compound **4** and by inactive compound **7**.

Conclusion

None of the imidazole derivatives tested shows remarkable antifungal activity. Structure–activity relation-

Table IV. Mutagenicity in the *Salmonella*-microsome test (plate incorporation procedure).

Compound	*Dose level (μg/plate)*	*His⁺ revertants/plate*							
		TA 1535		*TA 1537*		*TA 98*		*TA 100*	
		–S9	*+S9*	*–S9*	*+S9*	*–S9*	*+S9*	*–S9*	*+S9*
1	2000	11[a]	15	6	9	47	48	136	128
2	2000	12	15	13	11	43	59	137	116
3	10	26	23	10	12	74	64	**620**	**460**
4	10	19	16	9	14	70	62	**560**	**245**
5	20	27	10	10	10	**118**	75	**940**	**318**
6	200	**52**	**39**	7	12	78	55	**745**	**307**
7	2000	15	10	9	12	49	56	170	175
8	2000	12	10	12	12	43	49	160	149
Controls[b]									
Spontaneous		13	12	8	14	48	59	139	122
2AAF	50	12	17	7	13	45	**> 3000**	173	**> 3000**
EtBr	10	12	12	6	15	30	**> 2000**	151	153
MNNG	10	**> 5000**	**> 5000**	8	14	48	65	**> 5000**	**> 5000**
NaN_3	10	**> 2000**	**> 2000**	9	17	41	61	168	189

[a]Values are the average of different experiments. Positive results are indicated in bold. [b]2AAF, 2-acetylaminofluorene; EtBr, ethidium bromide; MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine.

ships reveal the genotoxicity of the imidazoles studied to be affected by the nature of the substituents. The lack of mutagenicity observed for compounds **1**, **2**, **7** and **8** is in agreement with the knowledge that the imidazole ring is a non-mutagenic structure [20]. Genotoxic activity is associated with the presence of the 'alerting' nitro group. The different substituents located at the 2-position were also found to modulate this activity. Of these, the thiomethyl group appears to confer higher mutagenic potency. The oxidation of the thiomethyl group retained mutagenicity, but compounds **4** and **5** were found to be less potent than the corresponding compound **3**. The presence of an additional 'alerting' group, the amino group (compound **6**), in substitution for the thiomethyl, did not enhance mutagenicity.

Experimental protocols

Chemistry

Melting points were determined on Buchi 510 apparatus and are uncorrected. The UV spectra were recorded on a Perkin Elmer Lambda 5 spectrophotometer using 1 cm quartz cells in a 10^{-5} M ethanol solution. The wavelength absorption maxima are reported in nanometers. The ^{1}H-NMR spectra were recorded with a Bruker AMX-400 WB (Centro Interdipartimentale Grandi Strumenti, Modena University) operating at 400.13 MHz. Chemical shifts are reported in ppm from tetramethylsilane used as internal standard, and are given in δ units. Microanalyses were carried out by R Gallesi in the Microanalysis Laboratory of the Dipartimento di Scienze Farmaceutiche, Modena University, and the analyses indicated by the symbols of the elements were within ±0.4% of the theoretical values. The compounds were separated by a Buchi model 685 (MPLC) glass column (460 × 36 mm ID), dry-filled with silica gel 60 (particle size 0.040–0.063 mm) (Merck) and connected to an LKB Multirac 2111 fraction collector. The fractions were monitored using 5 × 10 cm TLC plates.

The HPTLC analyses were conducted by means of a Linomat IV (Camag) spotter on 10 × 20 cm silica gel 60 F_{254} HPTLC plates and a Camag TLC Scanner II equipped with an Olivetti M280 PC operating the 'Cats 3.04' scanning program.

The solvents were analytical reagent grade; **1** was obtained from Sigma Chemical Co and was crystallized before utilization.

Syntheses

1-Methyl-2-methylsulfanyl-5-nitro-1H-imidazole **3**

Compound **2** (0.078 mol) [7], cooled to –5°C, was added to 1.054 mol of 65% nitric acid and 25 ml H_2O. The mixture was stirred in a steam bath for 60 min at 100°C and concentrated to a small volume. The residue was treated with ice and with sodium bicarbonate to pH 5, then collected and recrystallized from methanol to yield a yellow solid (4.9 g, 36%, mp 85–86°C, lit 86–88°C [21]). ^{1}H-NMR (DMSO-d_6) δ 2.77 (s, 3H, -SCH_3), 3.88 (s, 3H, N-CH_3), 8.24 (s, 1H, H-4); UV (ethanol) λ_{max} 348.1 (log ε 4.03).

Table V. Mutagenic activity of compounds **4** and **7** on *S cerevisiae* 6117 strain in the absence of microbial fraction.

Treatment time (h)	*Titre (cells/ml)*	*Survival*	*Gene conversion*			*Mitotic recombination*		
			Convertants/ ml	*Frequency*	*Treated/ control*	*Recombinants/ ml*	*Frequency*	*Treated/ control*
Control		N_c/N_{co}						
0	7.75×10^7	–	9.00×10^2	1.16×10^{-5}	–	1.20×10^4	1.55×10^{-4}	–
1	8.20×10^7	1.06	1.60×10^3	1.95×10^{-5}	1.68	9.00×10^3	1.10×10^{-4}	0.71
2	5.20×10^7	0.67	8.00×10^2	1.54×10^{-5}	1.33	9.10×10^3	1.75×10^{-4}	1.13
3	6.30×10^7	0.81	1.00×10^3	1.59×10^{-5}	1.37	7.60×10^3	1.21×10^{-4}	0.78
4	5.65×10^7	0.73	9.00×10^2	1.59×10^{-5}	1.37	8.60×10^3	1.52×10^{-4}	0.98
6	4.90×10^7	0.63	1.00×10^3	2.04×10^{-5}	1.76	8.40×10^3	1.71×10^{-4}	1.10
Compound 4, 20 µg/ml		N_t/N_c						
2	6.15×10^7	1.18	2.00×10^2	0.33×10^{-5}	0.21	1.24×10^4	2.00×10^{-4}	1.14
4	5.85×10^7	1.04	1.80×10^4	3.10×10^{-4}	**19.50**	8.60×10^3	1.47×10^{-4}	0.97
6	5.45×10^7	1.11	1.30×10^4	2.40×10^{-4}	**11.76**	3.80×10^3	0.70×10^{-4}	0.41
Compound 7, 5000 µg/ml		N_t/N_c						
2	4.75×10^7	0.91	1.20×10^3	2.53×10^{-5}	1.64	8.60×10^3	1.81×10^{-4}	1.03
4	5.15×10^7	0.91	1.00×10^3	1.94×10^{-5}	1.22	8.20×10^3	1.59×10^{-4}	1.05
6	6.80×10^7	1.39	1.80×10^3	2.65×10^{-5}	1.30	8.30×10^3	1.22×10^{-4}	0.71
MMS, 1100 µg/ml		N_t/N_c						
1	5.10×10^7	0.62	6.20×10^3	1.22×10^{-4}	**6.26**	1.02×10^4	2.00×10^{-4}	1.82
2	1.30×10^7	0.25	2.60×10^3	2.00×10^{-4}	**13.00**	5.40×10^3	4.15×10^{-4}	**2.37**
3	8.00×10^6	0.13	2.00×10^2	2.50×10^{-5}	1.57	3.84×10^3	4.80×10^{-4}	**3.97**

Positive results are indicated in bold. N_c: cells/ml at the different control times; N_{co}: cells/ml of control at time 0; N_t: cells/ml of treated at the different times; MMS: methyl methanesulfonate.

2-Methanesulfinyl-1-methyl-5-nitro-1H-imidazole **4** *and 2-methanesulfonyl-1-methyl-5-nitro-1H-imidazole* **5**

Compounds **4** and **5** were prepared by methods previously reported [7] for **7** and **8**, and separated on silica column with toluene/acetone (1:9, v:v).

4: (54%, recrystallized from acetone/petroleum ether 60–80°C, mp 94–95°C, lit 99°C [22]), ^{1}H-NMR ($CDCl_3$): δ 3.18 (s, 3H, $SOCH_3$); 4.28 (s, 3H, N-CH_3), 8.01 (s, 1H, H-4); UV (ethanol) λ_{max} 298.8 (log ε 3.98).

5: (75%, recrystallized from acetone/petroleum ether 60–80°C, mp 88–90°C, lit 91–92.5°C [21]), ^{1}H-NMR (DMSO-d_6) δ: 3.65 (s, 3H, SO_2CH_3), 4.28 (s, 3H, N-CH_3), 8.33 (s, 1H, H-4); UV (ethanol) λ_{max} 288.6 (log ε 3.96).

1-Methyl-5-nitro-1H-imidazol-2-ylamine **6**

Compound **6** was obtained in accordance with the method described in the literature [23].

6: (91%, recrystallized from acetone/petroleum ether 60–80°, mp 190°C, lit 205°C), ^{1}H-NMR (DMSO-d_6) δ 3.07 (s, 3H, N-CH_3), 8.01 (s, 1H, H-4), 7.25 (s, 2H, NH_2); UV (ethanol) λ_{max} 378.3 (log ε 4.08).

High performance thin-layer chromatography (HPTLC)

Compound **3** (5.8 mmol) was added to acetic acid in a 10 ml volumetric flask which was then filled to the mark. The solution was heated at 50 or 90°C and 2 or 4 equiv 30% hydrogen peroxide was added. Suitable volumes of standard solutions of **3**, **4** or **5** were spotted alternately on the HPTLC plates with 4 µl of a solution obtained by diluting with water 50 µl of reaction mixture in a 10 ml volumetric flask. The withdrawals were carried out at the following reaction times: 0, 5, 10, 15, 20, 30, 45, 60, and 90 min.

For the separation of **3** from **4** and **5**, the solvent system used was chloroform/*n*-hexane (3:2, v/v). All the plates were developed at room temperature using the ascending mode. The layers were analysed at 254 nm by the fluorescence-quenching method. The scanner was set up as follows: band width, 10 nm; span, 25; slit, 5 × 0.2 mm; scanning speed, 5 mm/s.

The identity of the compounds was determined by means of the R_f value and by a computerized identity check procedure, which confirmed the correlation of the sample spectra with the standard spectra. The calibration graphs were plotted for each plate using the linear regression equation obtained from the areas under the peaks for different amounts of standard solution.

X-ray diffraction analyses

1-Methyl-2-methylsulfanyl-5-nitro-1H-imidazole **3**

Molecular formula $C_5H_7N_3O_2S$; molecular weight 173.19; crystallized from methanol in the monoclinic space group $P2_1/n$

(No 14). The unit cell parameters were a = 7.765 (2), b = 6.571(1), c = 15.255(2) Å, β = 95.42(2)°, V = 774.9 Å^3, Z = 4, D_{calcd} = 1.484 g cm^{-3}, and $F(000)$ = 360. An orange-yellow prism of approximate dimensions 0.22 × 0.15 × 0.15 mm was used to collect intensity on an Enraf-Nonius CAD4 diffractometer, at room temperature using graphite-monochromated Mo$K\alpha$ radiation (λ = 0.71069 Å). Unit cell parameters were derived from least-squares fit to the setting angles of 25 intense reflections in the 7–14°θ range. Reflections were measured using the ω – 2θ scan mode with ω scan width (0.65 + 0.35 tanθ)° and ω scan speed 0.97–3.30° min^{-1}. Of 1578 reflections measured (1.5 ≤ θ ≤ 26°), 649 had $I \geq 3\ \sigma(I)$ and 603 were unique (R_{int} = 0.010) and were used, without absorption correction, (μ(Mo$K\alpha$) = 3.2 cm^{-1}) in the structure analysis. The structure was solved by direct methods using the SHELX86 program [25] and refined through full-matrix least-squares calculations by means of the SHELX76 program [26]. All non-hydrogen atoms were refined anisotropically, whereas the H atoms, located in ΔF maps, were refined isotropically through some least-squares cycles, and then held fixed because of the low reflection/parameter ratio. Final R and R_w values, with w from counting statistics, were 0.033 and 0.039 respectively.

2-Methanesulfinyl-1-methyl-5-nitro-1H-imidazole **4**

Molecular formula $C_5H_7N_3O_3S$; molecular weight 189.19; crystallized from acetone/petroleum ether 60–80°C in the monoclinic space group $P2_1/a$ (No 14). The unit cell parameters were a = 8.297 (1), b = 7.323 (1), c = 13.411 (2) Å, β = 97.84 (2), V = 807.2 (8) Å^3, Z = 4, D_{calcd} = 1.566 g cm^{-3} and $F(000)$ = 392. The yellow crystal selected for data collection had approximate dimensions of 0.35 × 0.28 × 0.20 mm. All X-ray measurements were carried out on an Enraf-Nonius CAD4 diffractometer at room temperature by using graphite-monochromated Mo$K\alpha$ radiation (λ = 0.71069 Å). Unit cell parameters were derived from least-squares fit to the setting angles of 25 intense reflections in the 10–19° θ range. Reflections were measured using the ω – 2θ scan mode with ω scan width (0.70 + 0.35 tan θ)° and ω scan speed 1.0–5.5°. Of 2017 reflections measured (1.5 ≤ θ ≤ 28°), 1514 had $I \geq 3\ \sigma(I)$ and 1447 were unique (R_{int} = 0.021). An empirical absorption correction based on the ψ scan [27] was applied to intensities (μ(Mo$K\alpha$) = 3.2 cm^{-1}, 0.946 ≤ T_{factor} ≤ 0.999). The structure was solved by direct methods [25], and was refined through full-matrix least-squares calculations [26], anisotropically for non-hydrogen atoms and isotropically for hydrogen atoms, to final R and R_w values of 0.036 and 0.042 respectively.

2-Methanesulfonyl-1-methyl-5-nitro-1H-imidazole **5**

Molecular formula $C_5H_7N_3O_4S$; molecular weight 205.19; crystallized from acetone/petroleum ether 60–80°C in the triclinic space group $P\bar{1}$ (No 2). The unit cell parameters are a = 9.034 (1), b = 9.298 (1), c = 11.593 (2) Å, α = 83.28 (2), β = 73.71 (2), γ = 66.23 (1)°, V = 855 (1) Å^3, Z = 4 and $F(000)$ = 424. All X-ray measurements were carried out on an Enraf-Nonius CAD4 diffractometer at room temperature by using graphite monochromated Mo$K\alpha$ radiation (λ = 0.71069 Å). The prismatic orange-yellow crystal selected for data collection had approximate dimensions of 0.25 × 0.18 × 0.18 mm, and was sealed into a glass capillary, since the compound is not air-stable. Unit cell parameters were derived from least-squares fit to the setting angles of 25 intense reflections in the 7–16° θ range. Reflections were measured using the ω – 2θ scan mode with ω scan width (0.60 + 0.35 tan θ)° and ω scan speed in the 0.9–4.1° min^{-1} interval. Of 3351 reflections measured (1.5 ≤ θ ≤ 26°), 1657 had $I \geq 3\ \sigma(I)$ and 1555 were unique (R_{int} = 0.014). The intensities of two standard reflections showed linear and almost isotropic crystal decay, *ca* 8.8% during 36 h of exposure time, which was corrected during data processing. Absorption correction was deemed unnecessary (μ(Mo$K\alpha$ = 3.1 cm^{-1}). The structure was solved by direct methods [25], and refined through full-matrix least-squares calculations [26]. All non-hydrogen atoms were refined anisotropically, whereas the hydrogen atoms, located in ΔF maps, were refined isotropically through some least-squares cycles, and then held fixed because of the low reflection/parameter ratio. Final R and R_w values were 0.031 and 0.032 respectively.

Final atomic coordinates for non-hydrogen atoms of compounds **3**, **4**, and **5** are given in tables VI, VII and VIII, respectively. Lists of positional and thermal parameters of hydrogen atoms, anisotropic temperature factors, bond distances and angles involving hydrogen atoms, torsion angles, selected least-squares planes, shortest nonbonded distances, and observed and calculated structure factors, are available on request from the author.

Table VI. Final fractional coordinates and equivalent isotropic temperature factors[a] for the 1-methyl-2-methylsulfanyl-5-nitro-1*H*-imidazole **3**.

Atom	*x*	*y*	*z*	B_{eq}, Å^2
N(1)	0.2649(4)	0.0567(5)	0.4609(2)	3.2(1)
C(2)	0.2876(5)	0.0906(6)	0.5481(3)	3.3(2)
N(3)	0.2248(5)	–0.0562(6)	0.5955(2)	4.0(2)
C(4)	0.1586(5)	–0.1929(7)	0.5358(3)	4.0(2)
C(5)	0.1814(5)	–0.1286(6)	0.4529(3)	3.4(2)
C(6)	0.3185(6)	0.1941(8)	0.3930(3)	5.1(2)
S	0.3918(2)	0.3089(2)	0.59102(8)	5.03(6)
C(7)	0.3716(7)	0.271(1)	0.7059(4)	7.1(3)
N(4)	0.1302(4)	–0.2291(6)	0.3730(3)	4.7(2)
O(3)	0.1581(5)	–0.1464(6)	0.3040(2)	7.1(2)
O(4)	0.0585(5)	–0.3943(5)	0.3774(3)	6.8(2)

[a] B_{eq} is defined as one-third of the trace of the orthogonalized B_{ij} tensor.

Biological assays

The compounds tested were dissolved or diluted in dimethylsulfoxide just before use.

Antifungal activity

The *in vitro* antifungal activity against yeasts and moulds was determined by the twofold serial dilution method [16] in liquid or solid nutrient medium. The minimum inhibitory concentration (MIC, μg/ml) was defined as the lowest dose level of compound which inhibits visible growth of fungi. The test was performed on Sabouraud dextrose broth (Oxoid) for *S cerevisiae* ATCC 9763, *C tropicalis* ATCC 1369, *A niger* ATCC 6275 and clinical isolates of *C albicans*, *C guilliermondii*, *C parapsilosis* and *C neoformans*. The compounds were tested in the concentration range from 25 to 200 μg/ml. The inoculum size added to the medium containing the antimycotic agents was 10^3 yeasts or spores/ml. MICs were determined by the observation of the cultures after 48 h incubation at 30°C. The antimycotic activity was also tested in Sabouraud agar against the following fungal strains isolated from clinical materials: *A flavus*, *A fumigatus*, *E floccosum*, *Microsporum gypseum*, *Pseudallescheria boydii*, *S schenckii*, *Trichophyton interdigitalis*, *T mentagro-*

Table VII. Final fractional coordinates and equivalent isotropic temperature factors[a] for the 2-methanesulfinyl-1-methyl-5-nitro-1*H*-imidazole **4**.

Atom	*x*	*y*	*z*	B_{eq}, Å^2
N(1)	0.8711(2)	0.4190(2)	0.7905(1)	3.29(6)
C(2)	0.9078(2)	0.3855(2)	0.6974(1)	2.88(6)
N(3)	0.9745(2)	0.5256(2)	0.6570(1)	3.73(6)
C(4)	0.9821(3)	0.6592(3)	0.7270(2)	4.48(9)
C(5)	0.9189(3)	0.5972(3)	0.8082(2)	4.15(8)
C(6)	0.7915(4)	0.2935(5)	0.8536(2)	5.1(1)
S	0.88181(5)	0.16425(6)	0.64091(3)	3.14(2)
O(1)	1.0491(2)	0.0849(2)	0.6472(1)	4.15(6)
C(7)	0.8263(3)	0.2416(3)	0.5148(1)	3.89(8)
N(4)	0.9003(4)	0.6952(4)	0.8984(2)	6.7(1)
O(3)	0.8520(4)	0.6155(6)	0.9675(2)	10.9(2)
O(4)	0.9431(5)	0.8526(4)	0.9013(2)	11.8(2)

[a] B_{eq} is defined as one-third of the trace of the orthogonalized B_{ij} tensor.

phytes, T rubrum, T soudanense, T violaceum. Of these, *Epidermophyton, Microsporum* and *Trichophyton* are moulds which cause dermatological infections (dermatophytoses). The compounds were tested in the 0.01–100 μg/ml range. The culture medium containing different concentrations of compound was transferred to Petri dishes. The inoculum, performed from exponentially growing cultures, was carried out by streaking the strains on the agar surface. Plates were then incubated at 25°C and observed periodically for 14 d. For comparative purposes miconazole was assayed as standard antifungal drug.

B subtilis rec-assay

The test is based on the differential growth inhibition produced on a pair of *B subtilis* strains: PB 1652 (*rec*$^+$; *lys*3, *met*B10, *trp*C2), able to repair DNA damage, and PB 1791 (*rec*$^-$; *met*B10, *trp*C2, *rec*E4), mutant defective in recombination repair. The *rec*-assay test was performed by the agar diffusion standard procedure [17]. Sterile filter paper disks measuring 9 mm in diameter, impregnated with a specific amount of test chemical in 30 μl DMSO, were placed on the center of Nutrient agar Petri plates seeded with 2 ml soft agar containing 10^8 *rec*$^+$ or *rec*$^-$ cells. Incubation was carried out at 37°C for 24 h, and then the diameter of the zone of inhibition around the disks was measured in mm. The chemical was considered a damaging agent when the ratio between the zone of inhibition of the repair deficient mutant *rec*$^-$ and that of the repair proficient *rec*$^+$ strain was greater than 1.2. The *rec*-assay with preincubation procedure was performed by including a preincubation at 4°C for 4 h before the incubation of the plates at 37°C. The *B subtilis rec*-assay performed in liquid medium was carried out by studying the antibacterial activity (minimum inhibitory concentration, MIC) against *rec*$^+$ and *rec*$^-$ strains. MIC was determined using the method of progressive double dilution. The substances were tested up to the maximum concentration of 1600 μg/ml in Mueller Hinton broth (Oxoid). The cultures of *B subtilis* strains were done in the same medium after overnight incubation at 37°C. The test tubes were inoculated with 10^4 *rec*$^+$ or *rec*$^-$ bacteria/ml and were incubated at 37°C. After 24 h, data were recorded. Compounds which showed increased antibacterial activity towards *rec*$^-$ strain (MIC value against *rec*$^-$ lower than that against *rec*$^+$ strain) were considered as DNA-damaging. DNA-damaging activity was detected by a *rec*$^+$/*rec*$^-$ MIC value higher than 1.

Salmonella-microsome test

Mutagenicity in the *Salmonella*-mammalian microsome test was determined by *S typhimurium* TA 1535, TA 1537, TA 98 and TA 100 strains. The test was performed using the plate-incorporation technique and the preincubation procedure with and without exogenous metabolic activation system [18]. Chemicals were tested at different concentrations and up to subtoxic doses. Following the plate incorporation method, 100 μl of an overnight culture of each strain (10^8 cells), various amounts of different compounds and, when required, 500 μl of metabolic activation system (S9-mix) were stirred with 2 ml of top agar supplemented with L-histidine and biotin. The mixture obtained was poured onto the surface of minimal glucose agar plates and incubated at 37°C for 72 h. The preincubation procedure was performed by adding in sterile capped tubes 100 μl of 1 M pH 7.4 phosphate buffer, different amounts of the sample solution, 100 μl of *S typhimurium* bacterial suspension (10^8 cells), 500 μl of S9-mix in the assay with metabolic activation, and sterile distilled water to a final volume of 1 ml. The tubes were incubated at 37°C for 30 min. Then 2 ml of molten top agar

Table VIII. Final fractional coordinates and equivalent isotropic temperature factors[a] for the 2-methanesulfonyl-1-methyl-5-nitro-1*H*-imidazole **5**.

Atom	*x*	*y*	*z*	B_{eq}, Å^2
N(1a)	0.0160(3)	0.4189(3)	0.8489(2)	2.9(1)
C(2a)	0.0770(4)	0.2795(4)	0.9029(3)	3.0(1)
N(3a)	–0.0393(3)	0.2425(3)	0.9821(3)	3.6(1)
C(4a)	–0.1854(4)	0.3654(4)	0.9769(3)	3.8(2)
C(5a)	–0.1540(4)	0.4729(4)	0.8967(3)	3.2(1)
C(6a)	0.1119(5)	0.4893(5)	0.7521(4)	4.5(2)
S(a)	0.2928(1)	0.1549(1)	0.86742(8)	3.21(4)
O(1a)	0.3794(3)	0.2337(3)	0.9036(2)	4.1(1)
O(2a)	0.3397(3)	0.1137(3)	0.7435(2)	4.5(1)
C(7a)	0.2964(5)	–0.0083(4)	0.9590(4)	4.6(2)
N(4a)	–0.2720(4)	0.6183(4)	0.8651(3)	3.9(1)
O(3a)	–0.2233(4)	0.7227(3)	0.8216(3)	5.3(1)
O(4a)	–0.4163(3)	0.6295(3)	0.8885(3)	5.1(1)
N(1b)	0.0959(3)	0.0965(3)	0.5859(3)	3.1(1)
C(2b)	–0.0685(4)	0.1683(4)	0.6418(3)	3.2(2)
N(3b)	–0.1203(4)	0.0936(4)	0.7382(3)	4.2(1)
C(4b)	0.0201(5)	–0.0355(4)	0.7462(4)	4.4(2)
C(5b)	0.1501(5)	–0.0354(4)	0.6551(3)	3.5(2)
C(6b)	0.1914(5)	0.1569(5)	0.4816(4)	5.1(2)
S(b)	–0.2037(1)	0.3513(1)	0.59603(8)	3.23(4)
O(1b)	–0.1968(3)	0.3379(3)	0.4730(2)	4.4(1)
O(2b)	–0.1592(3)	0.4704(3)	0.6262(2)	4.4(1)
C(7b)	–0.4004(4)	0.3675(4)	0.6857(4)	4.4(2)
N(4b)	0.3171(4)	–0.1506(4)	0.6313(4)	4.4(2)
O(3b)	0.4150(4)	–0.1483(3)	0.5350(3)	5.8(2)
O(4b)	0.3485(4)	–0.2489(3)	0.7101(3)	6.1(2)

[a] B_{eq} is defined as one-third of the trace of the orthogonalized B_{ij} tensor.

was added and the mixtures were overlaid on minimal glucose agar plates which were incubated at 37°C for 72 h. After incubation, revertant colonies were counted. Mutagenicity was detected by a dose-dependent increase in the number of revertants of three-fold background for TA 1535 and TA 1537 strains and two-fold for TA 98 and TA 100 strains. Spontaneous reversion was evaluated on control plates containing 100 µl of dimethylsulfoxide. 2-Acetylaminofluorene (2AAF), ethidium bromide (EtBr) (mutagens which require metabolic biotransformation), *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) and NaN_3 (direct-acting mutagens) were tested as positive controls in order to demonstrate that the test system was functional with known mutagens. The mutagenic potency of the active compounds was expressed by the number of revertants/µmol, calculated by the linear portion of the dose–response curve.

S cerevisiae mitotic segregation

This test was performed with *S cerevisiae* 6117 strain in the absence of metabolic activation [19]. Mitotic segregation produces colonies resistant to cycloheximide. These colonies are red when segregation is due to mitotic gene conversion, and white when segregation is due to somatic recombination or mitotic cross-over. For mutagenicity experiments the culture was centrifuged at 5000 rpm and the cells obtained, after washing with water, were suspended at 2×10^8 cells/ml in 20 mM pH 7.4 phosphate buffer. The test was performed by mixing 2.5 ml 20 mM pH 7.4 phosphate buffer, 500 µl of different concentrations of compound and 2 ml of cell suspension. The mixtures were incubated at 37°C. After 2, 4 and 6 h, 1 ml of each suspension was centrifuged at 5000 rpm, washed and suspended in 1 ml of sterile distilled water. The titre and the spontaneous and induced mitotic segregation of each suspension were then determined. After incubation at 30°C for 3–5 d, colonies were counted. Mutagenic compounds produced a number of induced segregants higher than that of spontaneous segregation and a response proportional to the concentrations and to the time of treatment. Inducibility of the mitotic segregation was checked with methyl methanesulfonate (MMS) after 1, 2 and 3 h of incubation at 30°C.

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