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Original article

Synthesis and biological evaluation of N-antipyrine-4-substituted amino-3-chloromaleimide derivatives

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ABSTRACT

This paper describes the synthesis of new cyclic imides obtained by reaction with N-antipyrine-3,4-dichloromaleimides and different aromatic amines. The analgesic activity of the synthesized compounds was initially investigated against the writhing test in mice, followed by analysis of the most promising compounds in this model and in the formalin-induced model. The results indicate that the compounds containing the electron-withdrawing substituents in the *para* position of the substitute ring exerted more potent analgesic activity in mice, being much more potent than the prototype N-antipyrine-3,4-dichloromaleimide and some reference drugs. Some compounds exhibited activity against human opportunistic and pathogenic fungi, with MIC values of between 40 and 100 μg/mL (91.74 and 236.96 μM), and it was verified that only a few compounds presented potential for cytotoxic activity.

1. Introduction

During our previous studies to achieve compounds with analgesic potential, it was verified that a new cyclic imide derivative (**1**) from 4-aminoantipyrine caused potent analgesic effects when analyzed against the writhing test in mice [1], but it also showed the toxicity signal.

The fragment 4-aminoantipyrine presents a certain similarity with metamizole (**2**), a well-known analgesic, anti-inflammatory and antipyretic agent, whose efficacy is unquestionable. Current studies suggest that the risk of agranulocytosis was exaggerated in the 1970's, and the risks from adverse reactions of metamizole are similar, or even lower, than for other analgesic/antipyretic drugs available on the market [2].

Based on these studies, a series of new cyclic imides obtained from 4-aminoantipyrine (**1**) were synthesized, particularly maleimide, succinimide, naphtalimide and glutarimide derivatives, and their analgesic potential evaluated in mice. The most promising compound, a 3,4-dichloromaleimide derivative (**3**), was analyzed using other models of nociception, and it was observed that this compound exerted potent analgesic activity in mice, being more active than some reference drugs [3] (Fig. 1).

We have recently observed considerable evidence that the derivative (**3**) produces anti-hypernociception in mice at the peripheral, spinal and supraspinal sites, and that interaction with group I metabotropic glutamate receptors (mGluRs) and N-methyl-D-aspartate (NMDA) receptors contributes to the mechanisms underlying its effect [4].

In order to obtain new therapeutic compounds which are structurally related to cyclic imides, and considering the high activity antinociceptive of derivative **3**, we have now synthesized and evaluated other analogues of this compound, keeping the imide fragments and antipyrine and therefore replacing one of the chlorines with different substituents.

In addition, given that some cyclic imides have antimicrobial effects, the compounds were screened by agar dilution, as an antibacterial and antifungal agent against a panel of human pathogens, to determine the minimum inhibitory concentration (MIC). Their cytotoxicity potential was also determined.

2. Results and discussion

2.1. Chemistry

Twenty-one compounds, being nineteen new compounds and two previously evaluated on mitochondrial effects by our group [5], were obtained from N-antipyrine-3,4-

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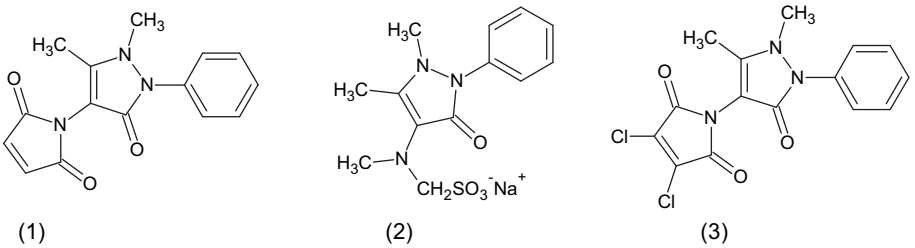


Fig. 1. Structures of N-antipyrinemaleimide (**1**), metamizole (**2**) and N-antipyrine-3,4-dichloromaleimide (**3**).

dichloromaleimide (**3**). It was obtained by the reaction of 3,4-dichloromaleic anhydride with aminophenazone (4-amino-antipyrine) in ether, and subsequently dehydrated by treatment with acetic acid/reflux to give the cyclic compound, as described previously [3]. This compound was used as a prototype and reacted with specific amines at a proportion of 1:2 under stirring, at room temperature. The substitution of only one chlorine atom probably occurs by a mechanism of addition–elimination, involving a tetrahedral intermediary, which loses a chlorine atom to form the desired product (Scheme 1). The substitution of the second chlorine atom by nucleophiles was not observed in the conditions studied. This can be attributed to the canonic structures, where the resonant structures indicate that a nucleophilic attack on the other olefinic carbon is unlikely [6].

The yields and melting points of the compounds are listed in Table 1. The chemical identification data (^{1}H NMR, ^{13}C NMR, IR and CHN) confirmed the structures of compounds **3**–**25**.

2.2. Evaluation of antinociceptive activity

In previous studies, compound **3** was evaluated using the acetic acid-induced writhing model, presenting an ID_{50} value of 10.8 (4.8–24.8) μmol/kg, being about 12-fold more potent than the standard drugs used as references in the same model [3]. The aim of this work was to obtain new and more active derivatives. All the new compounds were initially evaluated using the writhing test in mice, after intraperitoneal administration of a 10 mg/kg dose in a preliminary test. The results summarized in Table 1 reveal that the derivative compounds, with the exception of compounds **12** and **25**, showed a higher or similar antinociceptive effect when compared with non-steroidal drugs.

Among all the studied compounds, the most active were aniline derivatives with electron-withdrawing substituents in the *para* position. When compared with those of the *meta* position, it can be suggested that the electronic and steric effects contribute to the antinociception activity of these compounds. These hypotheses may be evidenced in the comparison between compounds **5** and **15**, which presented inhibitions of 75% and 39%, both with substitution of the chlorine atom in the *para* and *meta* positions, respectively. The contribution of electronegative substituents such as halogens, which have a balance between inductive electron-withdrawal and π–π conjugation, is extended to the more effective compounds in this series.

Although none of the replacements was more effective than compound **3** (inhibition of 99.0%), used as a prototype, the compounds with a halogen in the *para* position (**5**, **9**, **10** and **20**) were the most effective of the derivatives, and were therefore selected for more detailed studies in this test, to evaluate their potencies. As shown in Table 2, they exhibited notable activity, being about 33–284-fold more potent than the reference drugs ASA (acetyl salicylic acid) and MET (metamizole), and about 3–19-fold more potent than compound **3** itself.

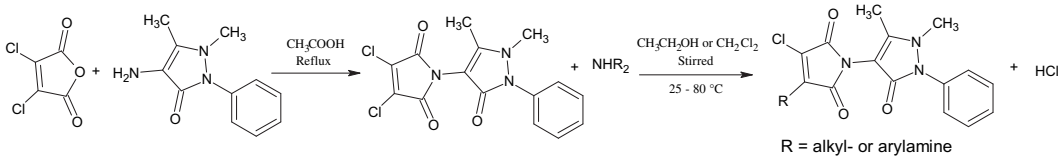
Since compounds **5**, **9**, **10** and **20** were the most active in the writhing test, these were selected for another model of pain: the formalin-induced pain model (Table 3). In this model ASA, an analgesic and anti-inflammatory drug, which is frequently used in therapeutic treatments, was inactive in preventing the first phase of formalin-induced (neurogenic) pain, and derivatives **5** and **10** presented signs of activity at a dosage of 10 mg/kg. In the second phase, ASA presented an ID_{50} value of 123.0 (77–209) μmol/kg, and derivative **20** presented inhibition of 49% with 20.5 μmol/kg (10 mg/kg), being about 6-fold more active than ASA.

The evaluation in the formalin test presents a distinctive biphasic nociception response termed the early and late phases. Drugs that act primarily on the central nervous system inhibit both phases equally, while peripherally acting drugs inhibit only the late phase. The early phase is probably a direct result of the stimulation of nociceptors in the paw, and reflects centrally mediated pain, while the late phase is due to inflammation with a release of serotonin, histamine, bradykinin and prostaglandins, and at least to some degree, to the sensitization of the central nociceptive neurons [7,8].

Besides the aniline derivatives, some other substituents were introduced and demonstrated interesting results. Compound **21**, with the broken conjugation between unsubstituted benzene and amine, showed less activity than compound **4** (aniline derivative unsubstituted). However, compound **22**, with two carbon atoms (phenethyl derivative unsubstituted), showed better activity than compound **4**. It is recognized that phenethyl derivatives increase narcotic analgesic potency in morphine analogues [9]. Such compounds with different substituents are being prepared, and the results will be shown later. Also, piperidine derivative **23** and piperazine derivative **24** demonstrated more activity than aniline derivative **4**.

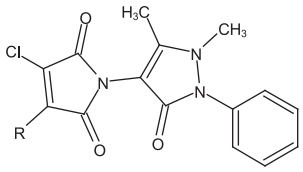
2.3. Evaluation of antimicrobial activities

The antimicrobial activity was determined by the agar dilution method. Our results indicate that some of the maleimide



Scheme 1. The synthesis of N-antipyrine-4-substituted amino-3-chloromaleimide derivatives.

Table 1
Structure and physical data and antinociceptive activity of N-antipyrine-3-4-dichloromaleimide derivatives and non-steroidal analgesic drugs (ASA and MET) given intraperitoneally at 10 mg/kg in mice in the writhing test.



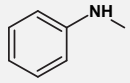
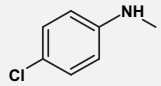
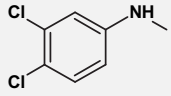
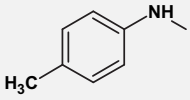
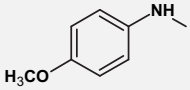
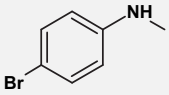
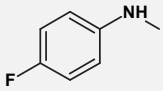
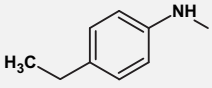
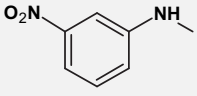
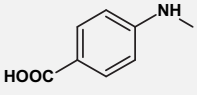
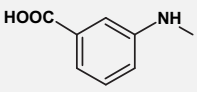
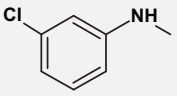
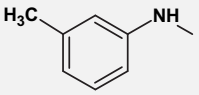
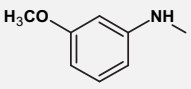
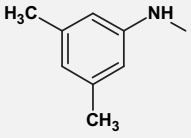
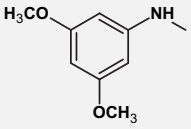
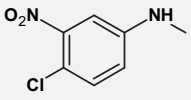
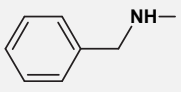
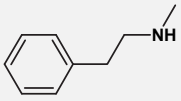
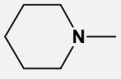
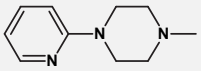
Compounds	Code	Reaction time (h)	Yield (%)	mp (°C)[a]	Inhibition (%)[b]
	**4**	3	63.0	266.3–268.4	71.6 ± 3.9**
	**5**	8	57.2	254.3–256.0	97.4 ± 0.4**
	**6**	9	60.9	253.0–254.8	75.0 ± 2.0**
	**7**	6	93.0	178.0–180.4	33.6 ± 3.7*
	**8**	6	95.0	208.8–209.5	31.2 ± 3.8*
	**9**	6	70.0	243.8–245.2	97.5 ± 0.6**
	**10**	6	80.0	263.8–265.0	93.2 ± 1.5**
	**11**	7	65.0	226.8–229.1	49.5 ± 2.9**
	**12**	10	60.0	118.2–120.0	29.5 ± 2.9*
	**13**	8	60.0	222.7–224.1	63.6 ± 3.3**
	**14**	8	68.0	224.8–226.0	33.9 ± 1.4*

Table 1 (*continued*)

Compounds	Code	Reaction time (h)	Yield (%)	mp (°C)[a]	Inhibition (%)[b]
	**15**	9	82.7	223.0–225.0	38.9 ± 4.4**
	**16**	7	52.0	136.5–138.7	49.7 ± 6.6**
	**17**	8	56.0	139.5–142.1	56.7 ± 3.1**
	**18**	7	61.0	137.8–139.9	68.4 ± 4.0**
	**19**	8	90.0	140.0–141.9	72.0 ± 2.0**
	**20**	9	82.0	121.9–123.2	98.5 ± 0.4**
	**21**	8	64.0	123.0–126.0	35.4 ± 3.6**
	**22**	1	54.8	157.7–159.0	81.8 ± 1.2**
	**23**	10	45.0	176.4–178.7	84.6 ± 1.3**
	**24**	10	70.0	132.2–133.0	77.7 ± 2.8**
	**25**	36	38.0	123.3–125.1	23.3 ± 2.8
Cl^-	**3**				99.0 ± 0.3**[c]
	ASA				35.0 ± 2.0*[c]
	MET				33.0 ± 3.5*[c]

[a] Melting points were uncorrected.
[b] Each group represents the mean of six experiments $^*p < 0.05$ and $^{**}p < 0.01$ compared with the corresponding control values.
[c] Campos et al., 2002.

Table 2
Effects of compounds **3**, **5**, **9**, **10**, **20**, ASA and MET on acetic acid-induced abdominal constrictions in mice.

Compound	ID_{50} (μmol/kg, i.p.)[a]	Inhibition (%)[b]
3	10.8 (4.8–24.4)	99.0 ± 1.0**
5	4.05 (2.26–7.25)	97.4 ± 0.4**
9	0.57 (0.37–0.92)	97.5 ± 0.6**
10	0.73 (0.52–1.03)	96.7 ± 0.7**
20	1.29 (0.96–1.74)	98.5 ± 0.4**
ASA	133 (73–243)	83 ± 1.4**
MET	162 (88–296)	54 ± 1.4**

Each group represents the mean ± SEM of six to eight animals. Compounds, acetyl salicylic (ASA) and metamizole (MET) were given intraperitoneally at 0.1–100 mg/kg.
[a] 95% confidence limit.
[b] $^{**}p < 0.01$ compared with the corresponding control value.

compounds tested exhibited activity, the fungi being selectively inhibited by compounds **4**, **6**, **9**, **10**, **11** and **21** (Table 4).

Analysis of the structures of the compounds did not enable a parameter of correlation with the antifungal activity to be established. However, compound **21** presented activity against some fungi. The main difference observed between this compound and the other active compounds (**4**, **6**, **9**, **10** and **11**) appeared to be related to the distance of a carbon between the imide ring and the aromatic ring, suggesting that the broken conjugation between the imide ring and the substituent group of the amino group, and consequently the hydrophobic factor, may be contributing to the antifungal activity. A similar effect has already been observed in previous studies [10,11].

Although previous studies have demonstrated that some cyclic imides present antibacterial activity [12–16], none of the tested compounds showed activity against bacteria at doses lower than 100 μg/mL. In other studies, activity was generally found at higher concentrations, as previously observed by Prado et al. [5]. For example, for the same compound **3** tested here, these authors found a MIC value of 250 μg/mL for antibacterial activity against *Staphylococcus aureus*. The maximum concentration value (100 μg/mL), was used as criteria because most of the clinically useful antibiotics are active against the tested strains at a level of at least 10 μg/mL. A compound which is not active until doses of at least 100 μg/mL is unlikely to be a serious candidate for clinical use.

2.4. Evaluation of cytotoxic activity

Through the brine shrimp lethality test, it was verified that the majority of the compounds tested were virtually non-cytotoxic (LC_{50} > 1000 μg/mL). Only four maleimides (**15**, **16**, **19** and **20**) presented potential for toxicity; compound **20** showed moderate toxicity to brine shrimp with LC_{50} = 94.15 μg/mL (192.81 μM), and seems to be related the presence of the electronic and hydrophobic nitro group, which is classified as being largely responsible for the toxicity of some drugs [17]. However, compounds **15** and **16** also presented moderate toxicity (LC_{50} = 86.23 and 90.54, respectively, equivalent to 194.52 and 214.11 μM), and compound **19** (LC_{50} = 248.68 μg/mL, equivalent to 532.64 μM) exhibited very low toxicity. For these compounds, it was not possible to establish a correlation with the steric, electronic and hydrophobic effects.

The compounds that presented cytotoxicity also presented substitution in the *meta* position of the aromatic ring. However, other compounds of this same series with substituents in this position were inactive. It is emphasized out of all active compounds, only compound **20** presented potential for toxicity. Moreover, future studies could be carried out with compounds **15**, **16** and **20** to evaluate the possible antitumoral activity, as it has been found that this test is predictive of this activity [18,19].

Table 3
Antinociceptive effects of compounds **9**, **10**, **20** and ASA against the first (0–5 min) and second (15–30 min) phases of formalin-induced licking.

Compound	Formalin test – Inhibition (%)	
	First phase	Second phase
5	28.2 ± 9.5*	29.3 ± 9.5
9	11.4 ± 4.5	22.5 ± 8.3
10	28.9 ± 5.9**	4.5 ± 9.0
20	19.6 ± 1.6	49.0 ± 9.2**
ASA	16.0 ± 2.0	45.0 ± 5.0*

Each group represents the mean ± SEM of six to eight animals. $^{*}p < 0.05$ and $^{**}p < 0.01$ compared with the corresponding control value.

Table 4
Antifungal activity of some compounds against pathogenic fungi.

Compound	Minimum inhibitory concentration (μg/mL)					
	A. fum[a]	*C. neo*[b]	*M. gyp*[c]	*Rhizopus*[d]	*T. men*[e]	*T. rub*[f]
04	>100	>100	>100	80	>100	>100
06	>100	>100	100	80	>100	>100
9	>100	>100	>100	100	>100	>100
10	>100	>100	100	>100	>100	>100
11	>100	>100	100	>100	>100	>100
21	100	40	>100	>100	100	100
Ketoconazole	7	5	6	15	8	3

[a] *Aspergillus fumigatus* (*A. fum*).
[b] *Cryptococcus neoformans* (*C. neo*).
[c] *Microsporum gypseum* (*M. gyp*).
[d] *Rhizopus sp* (*Rhizopus*).
[e] *Trichophyton mentagrophytes* (*T. men*).
[f] Trichophyton rubrum (T. rub).

3. Conclusion

In summary, the synthetic method enabled the preparation of 21 new N-antipyrine-4-substituted amino-3-chloromaleimide derivatives, with satisfactory to very good yields (37–95%). Four of these molecules (**5**, **9**, **10** and **20**) presented lower efficacy, but higher potency than prototype **3** and the positive control drugs (acetylsalicylic acid and metamizole), being all promising candidates for future analgesic drugs. The structure-activity analysis shows that the aromatic ring with electronegative substituent in the *para* position contributed to improving the analgesic action. The mechanism(s) of action by which these compounds exert analgesic activity still remain undetermined, and further studies are required to elucidate them. Also, the results indicate that some compounds (**4**, **6**, **9**, **10**, **11** and **21**), exhibited activity against pathogenic fungi, with MIC values of between 40 and 100 μg/mL (91.74 and 236.96 μM). Finally, through the cytotoxicity assay, it was verified that only compounds **15**, **16**, **19** and **20** presented potential for toxicity.

4. Experimental

4.1. General procedures for the synthesis of N-antipyrine-3,4 dichloromaleimide (*3*)

Compound **3** was obtained directly by the reaction of 3,4-dichloromaleic anhydride with 4-aminoantipyrine, followed by dehydrated in acetic acid under reflux, as previously described [3].

4.2. General procedures for the synthesis of compounds *4*–*25*

Compounds (**4**–**25**) were obtained by the reaction of the N-antipyrine-3,4-dichloromaleimide (0.057 mmol) [3], with appropriate

amines (0.057 mmol) in ethanol (15 mL), which were stirred overnight at room temperature. The product was filtered, but in cases where there was no precipitate, it was extracted with dichlorometane. The combined organic layers were dried with Na_2SO_4, filtered and evaporated. The product was purified by recrystallization with hexane and dichloromethane, or through chromatographic silica column gel, using chloroform:methanol as eluent.

4.3. Physicochemical data of the synthesized compounds

Melting points were determined with a Microquimica AP-300 apparatus (Florianópolis, Brazil) and were uncorrected. IR spectra were recorded with a Bomem 100 (Quebec, Canada) on KBr disks. Elemental analysis was determined with a Perkin Elmer 2400 (Norwalk, USA). Percentages of C and H were in agreement with the product formula (within ±0.4% of theoretical values). The ^{13}C and ^{1}H NMR spectra were recorded on a Brucker 200 MHz (Karlsruhe, Germany). The compounds were dissolved in deuterated solvents from commercial sources, with TMS as internal standard. The purity of the synthesized substances was monitored by thin-layer cromatography using Sigma (St. Louis, USA) silica pre-coated plastic plates of 200 μm in thickness, with various solvent systems of different polarities. Spots were visualized by short-wave UV light and iodine vapor. The spectral data (IR, ^{1}H and ^{13}C NMR), shown below, were in accordance with the structures.

4.3.1. N-antipyrine-3,4 dichloromaleimide (**3**)

Yield (%): 58.16; mp (°C): 208.2–209.2 °C; IR (KBr) ν (cm^{-1}): 1796; 1742 (ν –C=O, imide), 1642 (ν –C=O, antipyrine); ^{1}H NMR ($CDCl_3$) δ (ppm): 2.18 (s, 3H, CH_3); 3.21 (s, 3H, N–CH_3); 7.34–7.50 (m, 5H, Ar); ^{13}C NMR ($CDCl_3$) δ (ppm): 10.8 (CH_3); 35.2 (N–CH_3); 100.1 (=C–N); 125.1–134.2 (5 C, Ar); 127.7 (2 =C–Cl); 152.6 (=C–CH_3); 160.4 (2 C=O, imide); 161.8 (C=O, pyrazolone). Anal. Calcd. for $C_{15}H_{11}N_3O_3Cl_2$ (352.15 g/mol): C, 51.16; H, 3.15; N, 11.93. Found: C, 51.32; H, 3.22; N, 11.81%.

4.3.2. N-antipyrine-3-chloro-4-[aniline maleimide] (**4**)

Yield (%): 63%; mp (°C): 266.3–268.4 °C; IR (KBr) ν (cm^{-1}): 1781.8; 1722.6 (ν –C=O, imide); 1640.0 (ν –C=O, antipyrine); 3204.54 (ν –NH); ^{1}H NMR ($CDCl_3$) δ (ppm): 2.22 (*s*, 3H, CH_3); 3.22 (*s*, 3H, N–CH_3); 7.31–7.51 (*m*,5H, ar); ^{13}C NMR ($CDCl_3$) δ (ppm): 11.4 (CH_3); 35.1 (N–CH_3); 106.5 (=C–N); 109.3 (=C–Cl); 116.5–143.2 (12C, Ar); 127.0 (=C–N); 136.5 (=C–CH_3); 158.0 (C=O, imide); 161.3 (C=O, imide); 162.9 (C=O, pyrazolone). Anal. Calcd. for $C_{21}H_{17}N_4O_3Cl$ (408.7 g/mol); C, 61.69; H, 4.19; N, 13.70. Found: C, 61.02; H, 4.31; N, 13.43%.

4.3.3. N-antipyrine-3-chloro-4-[4-chloroaniline maleimide] (**5**)

Yield (%): 57.2%; mp (°C): 254.3–256.0 °C; IR (KBr) ν (cm^{-1}): 1780.1; 1727.3 (ν –C=O, imide); 1649.4 (ν –C=O, antipyrine); ^{1}H NMR ($CDCl_3$) δ (ppm): 2.21 (*s*, 3H, CH_3); 3.22 (*s*, 3H, N–CH_3); 7.10–7.40 (*m*,9H, ar); 8.09 (*s*, 1H, NH); ^{13}C NMR ($CDCl_3$) δ (ppm): 11.4 (CH_3); 35.1 (N–CH_3); 106.5 (=C–N); 109.3 (=C–Cl); 122.5 (C–Cl, Ar); 116.7–143.1 (11C, Ar); 127.0 (=C–N); 136.5 (=C–CH_3); 158.0 (C=O, imide); 161.3 (C=O, imide); 162.9 (C=O, pyrazolone). Anal. Calcd. for $C_{21}H_{16}N_4O_3Cl_2$ (474.21 g/mol): C, 56.90; H, 3.64; N, 12.64. Found: C, 56.41; H, 3.99; N, 12.01%.

4.3.4. N-antipyrine-3-chloro-4-[3,4dichloroaniline maleimide] (**6**)

Yield (%): 75%; mp (°C): 253.0–254.8 °C; IR (KBr) ν (cm^{-1}): 1781.5; 1722.7 (ν –C=O, imide), 1648.6 (ν –C=O, antipyrine); 3195.7 (ν –NH–); ^{1}H NMR ($CDCl_3$) δ (ppm): 2.10 (*s*, 3H, CH_3); 2.95 (*s*, 3H, N–CH_3); 6.75–7.70 (*m*,8H, ar); 13.9 (s, 1H, NH); ^{13}C NMR ($CDCl_3$) δ (ppm): 11.4 (CH_3); 35.1 (N–CH_3); 106.5 (=C–N); 109.3 (=C–Cl); 123.8 and 132.3(2C–Cl, Ar); 115.9–138.3 (11 C, Ar); 127.0 (=C–N); 136.5 (=C–CH_3); 158.0 (C=O, imide); 161.3 (C=O, imide); 162.9 (C=O, pyrazolone). Anal. Calcd. for $C_{21}H_{15}N_4O_3Cl_3$ (477.78 g/mol): C, 52.80; H, 3.16; N, 11.73. Found: C, 52.40; H, 3.49; N, 11.32%.

4.3.5. N-antipyrine-3-chloro-4-[4-methylaniline maleimide] (**7**)

Molecular formula (MW): $C_{22}H_{19}N_4O_4Cl_2$ (421.72 g/mol); Yield (%): 93%; mp (°C): 178.0–180.4 °C; IR (KBr) ν (cm^{-1}): 1781.5; 1726.4 (ν –C=O, imide); 1648.1 (ν –C=O, antipyrine); 3200.1 (ν –NH–); ^{1}H NMR ($CDCl_3$) δ (ppm): 2.21 (*s*, 3H, CH_3); 2.35 (*s*, 3H, N–CH_3); 3.20 (*q*, 2H, CH_2); 7.12–7.40 (*m*, 5H, ar); 7.69 (*s*, 1H, NH); ^{13}C NMR ($CDCl_3$) δ (ppm): 11.4 (CH_3); 20.5 (CH_3); 35.1 (N–CH_3); 106.5 (=C–N); 109.3 (=C–Cl); 118.0–144.2 (12 C, Ar); 127.0 (=C–N); 136.5 (=C–CH_3); 158.0 (C=O, imide); 161.3 (C=O, imide); 162.9 (C=O, pyrazolone). Anal. Calcd. for $C_{22}H_{19}N_4O_4Cl_2$ (421.72 g/mol): C, 62.49; H, 4.53; N, 13.25. Found: C, 62.67; H, 4.82; N, 12.98%.

4.3.6. N-antipyrine-3-chloro-4-[4-methoxyaniline maleimide] (**8**)

Molecular formula (MW): $C_{22}H_{19}N_4O_4Cl_2$ (438.72 g/mol); Yield (%): 95%; mp (°C): 208.8–209.5 °C; IR (KBr) ν (cm^{-1}): 1789.1; 1723.0 (ν –C=O, imide); 1639.8 (ν –C=O, antipyrine); 3200.1 (ν –NH–); ^{1}H NMR ($CDCl_3$) δ (ppm): 2.23 (*s*, 3H, CH_3); 3.24 (*s*, 3H, N–CH_3); 3.18 (*q*, 2H, CH_2); 3.81 (*q*, 2H, CH_2),7.40 (*m*,5H, ar); 8.09 (*s*, 1H, NH); ^{13}C NMR ($CDCl_3$) δ (ppm): 11.4 (CH_3); 35.1 (N–CH_3); 55.5 (OCH_3); 106.5 (=C–N); 109.3 (=C–Cl); 118.0–139.5 (11C, Ar); 127.0 (=C–N); 136.5 (=C–CH_3); 157.60 (C–OCH_3, Ar); 158.0 (C=O, imide); 161.3 (C=O, imide); 162.9 (C=O, pyrazolone). Anal. Calcd. for $C_{22}H_{19}N_4O_4Cl_2$ (438.72 g/mol): C, 60.21; H, 4.36; N, 12.77. Found: C, 60.32; H, 4.89; N, 12.41%.

4.3.7. N-antipyrine-3-chloro-4-[4-bromoaniline maleimide] (**9**)

Yield (%): 70%; mp (°C): 243.8–245.2 °C; IR (KBr) ν (cm^{-1}): 1781; 1726 (ν –C=O, imide); 1652 (ν –C=O, antipyrine); 3615 (ν –NH–); ^{1}H NMR ($CDCl_3$) δ (ppm): 2.18 (s, 3H, CH_3); 3.20 (s, 3H, N–CH_3); 6.97–7.49 (m, 9H, Ar); 8.47 (s, 1H, NH); ^{13}C NMR ($CDCl_3$) δ (ppm): 10.9 (CH_3); 35.1 (N–CH_3); 95.3 (=C–N); 100.7 (=C–Cl); 118.7 (C–Br, Ar); 125.2–152.4 (11C, Ar); 135.2 (=C–N); 137.4 (=C–CH_3); 160.7 (C=O, imide); 164.7 (C=O, imide); 166.1 (C=O, pyrazolone). Anal. Calcd. for $C_{21}H_{16}Br\ Cl\ N_4O_3$(487.73 g/mol): C, 51.71; H, 3.31; N, 11.49. Found: C, 51.62; H, 3.56; N, 11.33%.

4.3.8. N-antipyrine-3-chloro-4-[4-fluoroaniline maleimide] (**10**)

Yield (%): 80%; mp (°C): 263.8–265.0 °C; IR (KBr) ν (cm^{-1}): 1783; 1725 (ν –C=O, imide); 1658 (ν –C=O, antipyrine); 3619 (ν –NH–); ^{1}H NMR ($CDCl_3$) δ (ppm): 2.20 (s, 3H, CH_3); 3.21 (s, 3H, N–CH_3); 6.99–7.46 (m, 9H, Ar); 7.77 (s, 1H, NH); ^{13}C NMR ($CDCl_3$) δ (ppm): 11.0 (CH_3); 35.3 (N–CH_3); 94.3 (=C–N); 101.1 (=C–Cl); 115.3 e 115.7 (CH, Ar–F); 125.9 e 126.0 (CH, Ar–F); 125.2–134.0 (6C, Ar); 131.7 (=C–N); 137.7 (=C–CH_3); 152.6 (C–NH, Ar); 158.4 e 163.5 (C–F, Ar); 160.8 (C=O, imide); 164.8 (C=O, imide); 166.1 (C=O, pyrazolone). Anal. Calcd. for $C_{21}H_{16}F\ Cl\ N_4O_3$ (426.82 g/mol): C, 59.09; H, 3.78; N, 13.13. Found: C, 59.63; H, 3.92; N, 12.98%.

4.3.9. N-antipyrine-3-chloro-4-[4-ethylaniline maleimide] (**11**)

Yield (%): 65%; mp (°C): 226.8–229.1 °C; IR (KBr) ν (cm^{-1}): 1750; 1727 (ν –C=O, imide), 1659 (ν –C=O, antipyrine); 3176 (ν –NH–); ^{1}H NMR ($CDCl_3$) δ (ppm): 1.17 (t, 3H, CH_3), 2.15 (s, 3H, CH_3), 2.58 (q, 2H, CH_2); 3.16 (s, 3H, N–CH3), 7.0–7.40 (m, 9H, Ar); 8.80 (s, 1H, NH); ^{13}C NMR ($CDCl_3$) δ (ppm): 11.0 (CH_3); 15.7 (CH_3–Et); 28.6 (CH_2); 35.3 (N–CH_3); 94.0 (=C–N); 101.1 (=C–Cl); 114.6 (=C–NH); 124.2–142.7 (11C, Ar); 137.8 (=C–CH_3); 152.54 (C–Et, Ar), 160.6 (C=O, imide); 165.3 (C=O, imide); 166.6 (C=O, pyrazolone). Anal. Calcd. for $C_{23}H_{21}Cl\ N_4O_3$ (436.89 g/mol): C, 63.23; H, 4.84; N, 12.82. Found: C, 63.51; H, 4.98; N, 12.65%.

*4.3.10. N-antipyrine-3-chloro-4-[3-nitroaniline maleimide] (**12**)*

Yield (%): 60%; mp (°C): 118.2–120.0 °C; IR (KBr) ν (cm^{-1}): 1750; 1727 (ν –C=O, imide), 1663 (ν –C=O, antipyrine); 3290 (ν –NH–); ^{1}H NMR ($CDCl_3$) δ (ppm): 2.26 (s, 3H, CH_3); 3.23 (s, 3H, N–CH_3); 7.36–8.04 (m, 9H, Ar); 10.32 (s, 1H, NH); ^{13}C NMR ($CDCl_3$) δ (ppm): 10.2 (CH_3); 35.1 (N–CH_3); 95.9 (=C–N); 98.9 (=C–Cl); 117.6–138.1 (11 CH, Ar); 138.2 (=C–CH_3); 147.5 (=C–N); 154.2 (C–NO_2, Ar); 160.6 (C=O, imide); 164.5 (C=O, imide); 166.7 (C=O, pyrazolone). Anal. Calcd. for $C_{21}H_{16}Cl\ N_5O_5$ (453.83 g/mol): C, 55.58; H, 3.55; N, 15.43. Found: C, 55.76; H, 3.95; N, 15.06%.

*4.3.11. N-antipyrine-3-chloro-4-[4-carboxyaniline maleimide] (**13**)*

Yield (%): 60%; mp (°C): 222.7–224.1 °C; IR (KBr) ν (cm^{-1}): 1750; 1728 (ν –C=O, imide), 1653 (ν –C=O, antipyrine); 3316 (ν –NH–); ^{1}H NMR ($CDCl_3$) δ (ppm): 2.30 (s, 3H, CH_3); 3.08 (s, 3H, CH_3); 6.30–6.85 (m, 9H, Ar); 8.60 (s, 1H, NH); 8.69 (s, 1H, COOH); ^{13}C NMR ($CDCl_3$) δ (ppm): 10.3 (CH_3); 35.1 (N–CH_3); 93.6 (=C–N); 99.2 (=C–Cl); 122.7–134.3 (11C, Ar); 137.0 (=C–CH_3); 141.0 (=C–N); 154.2 (C–COOH, Ar); 160.6 (C=O, imide); 164.6 (C=O, imide); 165.9 (C=O, pyrazolone), 166.9 (C=O, COOH). Anal. Calcd. for $C_{22}H_{17}Cl\ N_4O_5$ (452.84 g/mol): C, 58.35; H, 3.78; N, 12.37. Found: C, 58.27; H, 3.97; N, 12.62%.

*4.3.12. N-antipyrine-3-chloro-4-[3-carboxyaniline maleimide] (**14**)*

Yield (%): 68%; mp (°C): 224.8–226.0 °C; IR (KBr) ν (cm^{-1}): 1780; 1719 (ν –C=O, imide), 1653 (ν –C=O, antipyrine); 3309 (ν –NH–), 2531 (ν –COOH–); ^{1}H NMR ($CDCl_3$) δ (ppm): 2.26 (s, 3H,CH_3); 3.23 (s, 3H, N–CH_3), 7.37–7.79 (m, 9H, Ar); 10.15 (s, 1H, COOH); ^{13}C NMR ($CDCl_3$) δ (ppm): 10.2 (CH_3); 35.1 (N–CH_3); 93.6 (=C–N); 99.1 (=C–Cl); 124.4–154.3 (11C, Ar); 128.5 (=C–NH); 130.9 (C–COOH, Ar); 138.5 (=C–CH_3, Ar); 160.6 (C=O, imide); 164.6 (C=O, imide); 165.9 (C=O, pyrazolone); 166.8 (C=O, COOH). Anal. Calcd. for $C_{22}H_{17}Cl\ N_4O_5$ (452.84 g/mol): C, 58.35; H, 3.78; N, 12.37. Found: C, 58.29; H, 3.95; N, 12.61%.

*4.3.13. N-antipyrine-3-chloro-4-[3-chloroaniline maleimide] (**15**)*

Yield (%): 82.75%; mp (°C): 223.0–226.0 °C; IR (KBr) ν (cm^{-1}): 1779; 1726 (ν –C=O, imide), 1645 (ν –C=O, antipyrine); 3950 (ν –NH–); ^{1}H NMR ($CDCl_3$) δ (ppm): 2.15 (s, 3H, CH_3), 3.19 (s, 3H, N–CH_3), 7.0–7.45 (m, 9H, Ar); 8.58 (s, 1H, NH); ^{13}C NMR ($CDCl_3$) δ (ppm): 10.9 (CH_3); 35.1 (N–CH_3); 96.1 (=C–N); 100.9 (=C–Cl); 121.6–152.6 (11C, Ar); 127.6 (=C–NH); 137.1 (C–Cl, Ar); 137.3 (=C–CH_3); 160.8 (C=O,imide); 164.7 (C=O, imide); 165.9 (C=O, pyrazolone). Anal. Calcd. for $C_{21}H_{16}Cl_2N_4O_3$ (443.28 g/mol): C, 56.90; H, 3.64; N, 12.64. Found: C, 57.03; H, 3.87; N, 12.41%.

*4.3.14. N-antipyrine-3-chloro-4-[3-methylaniline maleimide] (**16**)*

Molecular formula (MW): $C_{22}H_{19}Cl\ N_4O_3$ (422.86 g/mol); Yield (%): 52%; mp (°C): 136.5–138.7 °C; IR (KBr) ν (cm^{-1}): 1782; 1725 (ν –C=O, imide), 1658 (ν –C=O, antipyrine); 3617 (ν –NH–); ^{1}H NMR ($CDCl_3$) δ (ppm): 2.20 (s, 3H, CH_3), 2.35 (s, 3H, CH_3–ar), 3.19 (s, 3H, N–CH_3), 6.99–7.48 (m, 9H, Ar), 7.71 (s, 1H, NH); ^{13}C NMR ($CDCl_3$) δ (ppm): 10.9 (CH_3); 21.4 (CH_3–Ar); 35.3 (N–CH_3); 94.3 (=C–N); 101.2 (=C–Cl); 120.8–138.6 (11C, Ar); 134.2 (=C–NH); 135.6 (C–N, Ar); 137.4 (=C–CH_3); 152.9 (C–CH_3, Ar); 160.8 (C=O, imide); 165.0 (C=O, imide); 166.3 (C=O, pyrazolone). Anal. Calcd. for $C_{22}H_{19}Cl\ N_4O_3$ (422.86 g/mol): C, 62.49; H, 4.53; N, 13.25. Found: C, 62.53; H, 4.78; N, 13.11%.

*4.3.15. N-antipyrine-3-chloro-4-[3-methoxyaniline maleimide] (**17**)*

Yield (%): 56%; mp (°C): 139.5–142.1 °C; IR (KBr) ν (cm^{-1}): 1781; 1726 (ν –C=O, imide), 1658 (ν –C=O, antipyrine); 3639 (ν –NH–); ^{1}H NMR ($CDCl_3$) δ (ppm): 2.20 (s, 3H,CH_3); 3.20 (s, 3H, N–CH_3), 3.81 (s, 3H, OCH_3), 6.74–7.49 (m, 9H, Ar); 7.87 (s, 1H, NH); ^{13}C NMR ($CDCl_3$) δ (ppm): 10.9 (CH_3); 35.3 (N–CH_3); 55.3 (OCH_3); 94.7 (=C–N); 101.2 (=C–Cl); 109.2–152.8 (11C, Ar); 134.1 (=C–NH); 137.3 (=C–CH_3); 159.8 (C–OCH_3, Ar); 160.8 (C=O, imide); 165.0 (C=O, imide); 166.3 (C=O, pyrazolone). Anal. Calcd. for $C_{22}H_{19}Cl\ N_4O_4$ (438.86 g/mol); C, 60.21; H, 4.36; N, 12.77. Found: C, 60.34; H, 4.54; N, 12.59%.

*4.3.16. N-antipyrine-3-chloro-4-[3,5-dimethylaniline maleimide] (**18**)*

Molecular formula (MW): $C_{23}H_{21}Cl\ N_4O_3$ (436.89 g/mol); Yield (%): 61%; mp (°C): 137.8–139.9 °C; IR (KBr) ν (cm^{-1}): 1781; 1725 (ν –C=O, imide), 1661 (ν –C=O, antipyrine); 3300 (ν –NH–); ^{1}H NMR ($CDCl_3$) δ (ppm): 2.21 (s, 3H, CH_3); 3.20 (s, 3H, N–CH_3); 2.32 (s, 6H, CH_3); 6.80–4.76 (m, 8H, Ar); ^{13}C NMR ($CDCl_3$) δ (ppm): 11.0 (CH_3); 35.3 (N–CH_3); 21.3 (2 CH_3); 94.2 (=C–N); 101.4 (=C–Cl); 121.4–152.9 (11C, Ar); 134.21 (=C–N); 137.4 (=C–CH_3); 160.8 (C=O, imide); 165.1 (C=O, imide); 166.3 (C=O, pyrazolone). Anal. Calcd. for $C_{23}H_{21}Cl\ N_4O_3$ (436.89 g/mol): C, 63.23; H, 4.84; N, 12.82. Found: C, 63.31; H, 4.98; N, 12.67%.

*4.3.17. N-antipyrine 3-chloro-4-[3,5-dimethoxyaniline maleimide] (**19**)*

Yield (%): 90%; mp (°C): 140.0–141.9 °C; IR (KBr) ν (cm^{-1}): 1780; 1726 (ν –C=O, imide), 1653 (ν –C=O, antipyrine); 3360 (ν –NH–); ^{1}H NMR ($CDCl_3$) δ (ppm): 2.19 (s, 3H, CH_3); 3.19 (s, 3H, N–CH_3); 3.77 (s, 6H, OCH_3); 6.32 (s, CH, Ar); 7.25–7.45 (m, 9H, Ar); 7.74 (s, 1H, NH); ^{13}C NMR ($CDCl_3$) δ (ppm): 10.9 (CH_3); 35.2 (N–CH_3); 55.5 (2 OCH_3); 95.0 (=C–N); 98.5–137.4 (10C, Ar); 101.0 (=C–Cl); 137.2 (=C-CH_3); 152.6 (C=O, imide); 160.6 (2 C–OCH_3); 165.0 (C=O, imide); 166.2 (C=O, pyrazolone). Anal. Calcd. for $C_{23}H_{21}Cl\ N_4O_5$ (466.88 g/mol): C, 58.91; H, 4.51; N, 11.95. Found: C, 58.96; H, 4.76; N, 11.82%.

*4.3.18. N-antipyrine-3-chloro-4-[4-chloro-3-nitroaniline maleimide] (**20**)*

Yield (%): 82%; mp (°C): 121.9–123.2 °C; IR (KBr) ν (cm^{-1}): 1798; 1727 (ν –C=O, imide), 1658 (ν –C=O, antipyrine); 3439 (ν –NH–); ^{1}H NMR ($CDCl_3$) δ (ppm): 2.23 (s, 3H, CH_3); 3.21 (s, 3H, N–CH_3); 7.35–7.87 (m, 10H, Ar); 10.32 (s, 1H, NH); ^{13}C NMR ($CDCl_3$) δ (ppm): 10.9 (CH_3); 35.8 (N–CH_3); 97.8 (=C–N); 99.5 (=C–Cl); 120.4–147.6 (10 C, Ar); 127.8 (=C–N); 132.1 (C–Cl, Ar); 138.6 (=C–CH_3); 154.8 (C–NO_2, Ar); 161.2 (C=O, imide); 165.1 (C=O, imide); 166.2 (C=O, pyrazolone). Anal. Calcd. for $C_{21}H_{16}Cl_2\ N_5O_5$ (488.28 g/mol): C, 51.66; H, 3.10; N, 14.34. Found: C, 51.82; H, 3.31; N, 14.13%.

*4.3.19. N-antipyrine 3-chloro-4-[benzylamine maleimide] (**21**)*

Yield (%): 64%; mp (°C): 123.0–126.0 °C; IR (KBr) ν (cm^{-1}): 1750; 1726 (ν –C=O, imide); 1662 (ν –C=O, antipyrine); 3285 (ν –NH–); ^{1}H NMR ($CDCl_3$) δ (ppm): 2.19 (s, 3H, CH_3); 3.19 (s, 3H, N–CH_3); 4.84 (d, 2H, CH_2); 7.34–7.48 (m,10H, Ar); 5.80 (s, 1H, NH); ^{13}C NMR ($CDCl_3$) δ (ppm): 11.2 (CH_3); 35.6 (N––CH_3); 47.4 (CH_2); 125.1 (=C–N); 125.1 (=C–Cl); 126.7 (=C–NH); 127.7–137.1 (12 C, Ar); 140.8 (=C–CH_3); 153.1 (C=O, imide); 164.7 (C=O, imide); 166.4 (C=O, pyrazolone). Anal. Calcd. for $C_{22}H_{19}Cl\ N_5O_5$ (422.86 g/mol): C, 62.49; H, 4.53; N, 13.25. Found: C, 62.57; H, 4.67; N, 13.08%.

*4.3.20. N-antipyrine 3-chloro-4-[phenethylamine maleimide] (**22**)*

Yield (%): 54.8%; mp (°C): 157.7–159.0 °C; IR (KBr) ν (cm^{-1}): 1725; 1661 (ν –C=O, imide); 1623 (ν –C=O, antipyrine); 3297 (ν –NH–); ^{1}H NMR ($CDCl_3$) δ (ppm): 2.21 (s, 3H, CH_3); 2.76 (t, 2H, CH_2); 3.07 (s, 3H, N–CH_3); 3.34 (t, 2H, CH_2); 7.21–7.58 (m, 10 H, Ar); 9.71 (s, 1H, NH); ^{13}C NMR ($CDCl_3$) δ (ppm): 10.9 (CH_3); 34.5 (N–CH_3); 35.8 (CH_2); 41.1 (CH_2); 105.9 (=C–Cl); 123.7–152.7 (12C, Ar); 126.2 (=C–N); 126.4 (=C–NH); 139.1 (=C–CH_3); 160.2 (C=O,

imide); 160.4 (C═O, imide); 161.3 (C═O, pyrazolone). Anal. Calcd. for $C_{23}H_{21}Cl\ N_4O_3$ (436.90 g/mol): C, 63.23; H, 4.84; N, 12.82. Found: C, 63.31; H, 4.99; N, 12.73%.

4.3.21. N-antipyrine 3-chloro-4-[piperidine maleimide] (**23**)

Yield (%): 45%; mp (°C): 176.4–178.7 °C; IR (KBr) ν (cm^{-1}): 1724 (ν –C═O, imide), 1655 (ν –C═O, antipyrine); 3397 (ν –NH–); ^1H NMR ($CDCl_3$) δ (ppm): 1.71 (m, 6H, CH_2); 2.18 (s, 3H, CH_3); 3.18 (s, 3H, N–CH_3); 3.91 (m, 4H, CH_2); 7.27–7.48 (m, 5H, Ar); ^{13}C NMR ($CDCl_3$) δ (ppm): 11.0 (CH_3); 24.1 (CH_2); 26.7 (CH_2); 35.4 (N–CH_3); 49.7 (CH_2); 94.1 (═C–Cl); 102.0 (═C–N); 124.7–142.1 (6C, Ar); 134.4 (═C–N); 153.1 (═C–CH_3); 160.9 (C═O, imide); 164.5 (C═O, imide); 165.2 (C═O, pyrazolone). Anal. Calcd. for $C_{20}H_{21}Cl\ N_4O_3$ (400.85 g/mol): C, 59.92; H, 5.28; N, 13.98. Found: C, 60.01; H, 5.42; N, 13.74%.

4.3.22. N-antipyrine 3-chloro-4-[(pyridin-2-yl)piperazine maleimide] (**24**)

Yield (%): 70%, mp (°C): 132.2–133.0 °C; IR (KBr) ν (cm^{-1}): 1714; 1657 (ν –C═O, imide); 1625 (ν –C═O, antipyrine); ^1H NMR ($CDCl_3$) δ (ppm): 2.19 (s, 3H, CH_3); 3.19 (s, 3H, N–CH_3); 3.70 (dd, 2H, CH_2); 4.12 (dd, 2H, CH_2); 6.66–8.22 (m, 9H, Ar); ^{13}C NMR ($CDCl_3$) δ (ppm): 11.0 (CH_3); 35.4 (N–CH_3); 45.6 (CH_2); 47.7 (CH_2); 95.9 (═C–N); 101.7 (═C–Cl); 107.3–158.6 (11C, Ar); 134.4 (C–N); 153.1 (═C–CH_3); 160.9 (C═O, imide); 164.5 (C═O, imide); 165.0 (C═O, pyrazolone). Anal. Calcd. for $C_{24}H_{23}Cl\ N_6O_3$ (478.94 g/mol): C, 60.19; H, 4.84; N, 17.55. Found: C, 60.32; H, 4.97; N, 17.35%.

4.3.23. N-antipyrine 3-chloro-4-[1H-pyrrol-2-yl maleimide] (**25**)

Molecular formula (MW): $C_{19}H_{15}ClN_4O_3$ (382.80 g/mol); Yield (%): 36.8%; mp (°C): 123.3–125.1 °C; IR (KBr) ν (cm^{-1}): 3399 (ν –NH–), 1718; 1713 (ν –C═O, imide), 1656 (ν –C═O, antipyrine); ^1H NMR ($CDCl_3$) δ (ppm): 2.19 (s, 3H, CH_3); 3.21 (s, 3H, N–CH_3); 6.42–7.50 (m, 8H, Ar); 10.49 (s, 1H, NH); ^{13}C NMR ($CDCl_3$) δ (ppm): 10.9 (CH_3); 35.3 (N–CH_3); 101.0 (═C–N); 112.1 (CH, pyrrole); 117.9 (CH, pyrrole); 118.5 (CH, pyrrole); 121.4 (C–Cl); 124.9–129.3 (6C, Ar); 134.1 (═C–CH_3); 152.8 (C–pyrrole); 160.7 (C═O, imide); 164.8 (C═O, imide); 168.6 (C═O, pyrazolone). Anal. Calcd. for $C_{19}H_{15}ClN_4O_3$ (382.80 g/mol): C, 59.61; H, 3.95; N, 14.64. Found: C, 59.84; H, 4.11; N, 14.39%.

4.4. Biological assay

4.4.1. Animals

Swiss mice (25–35 g) were obtained from the Animal House of the University of Vale do Itajaí (Itajaí, Brazil). The animals were housed in automatically controlled temperature conditions (23 ± 2 °C and 12 h light–dark cycles). They were given access to water and Nuvital chow *ad libitum*, and remained in the appropriate laboratory at UNIVALI until several hours before the experiments. The allocation of animals into the different groups was randomized, and the experiments were carried out under blind conditions. Since some suffering could result from the experiments, the guidelines of the IASP Committee on Ethical Issues were followed [20].

4.4.2. Acetic acid-induced writhing

The abdominal constriction response caused by intraperitoneal injection of diluted acetic acid (0.6%) was determined according to the procedures described previously, with minor modifications [21,22]. The animals were pretreated with 10 mg/kg of N-antipyrine-3,4-dichloromaleimide derivatives (**4**–**25**) or standard drugs via the intraperitoneal route, 30 min before acetic acid injection. The control animals received a similar volume of vehicle (Tween 80 and 0.9% NaCl) (10 ml/kg, i.p.). All the experiments were carried out at 20–22 °C. After the challenge, each animal was placed in a separate glass funnel, and the number of abdominal constrictions, together with stretching of the hind limbs, was cumulatively counted over a period of 20 min. For compounds **5**, **9**, **10** and **20**, the abdominal constriction response was analyzed, following pretreatment via the intraperitoneal route in smaller doses (0.1–10 mg/kg) and for standard drugs at higher doses (10–100 mg/kg) for evaluation of ID_{50}.

4.4.3. Formalin test

The observation chamber was a glass cylinder of 20 cm in diameter, with a mirror at a 45° angle to allow clear observation of the animal's paws. The mice were treated with vehicle solution (i.p.), compounds **5**, **9**, **10** and **20** and ASA (10 mg/kg, i.p.), 30 min before formalin injection. Each animal was placed in the chamber for 5 min before treatment, in order to acclimatize to the new environment. The formalin test was carried out as described by Hunskaar et al. [23], with minor modifications. Twenty microliters of a 2.5% formalin solution (0.92% formaldehyde) in 0.9% saline solution were injected intraplantarly into the right hind paw. The animal was then returned to the chamber, and the amount of time spent licking the injected paw was considered indicative of pain. Two distinct phases of intensive licking activity were identified: an early acute phase and a late or tonic phase (0–5 and 15–30 min after formalin injection, respectively).

4.4.4. Statistical analysis

The results are presented as mean ± S.E.M., except for the mean ID_{50} values (i.e. the dose of drugs or compounds that reduces the antinociceptive response by 50% relative to the control value) which are reported as geometric means accompanied by their respective 95% confidence limits. The statistical significance between the groups was determined by analysis of variance, followed by Dunnett's multiple comparison test. *P*-values of less than 0.05 were considered indicative of significance. The ID_{50} values were determined by graphical interpolation from the individual experiments.

4.4.5. Drugs

The following drugs were used: ASA, acetaminophen, metamizole (all from Sigma Chemical) and acetic acid (E. Merck). All the compounds were dissolved in Tween 80 (E. Merck), plus 0.9% of NaCl solution. The final concentration of Tween 80 did not exceed 5% and did not cause any effect *per se*.

4.5. Antimicrobial analysis

4.5.1. Microorganisms

For the antimicrobial evaluation, standardized strains of the following bacteria were used, obtained from the American Type Culture Collection (ATCC) Rockville, MD, USA, and CEREMIC (C), Centro de Referencia Micológica, Facultad de Ciencias Bioquímicas y Farmacéuticas, Rosario, Argentina, and Control Lab (CL), Rio de Janeiro, Brazil: *Bacillus cereus* ATCC 14579, *Bacillus subtilis* ATCC 23858, *Enterobacter cloacae* ATCC 35030, *Escherichia coli* ATCC 11775, *Proteus mirabilis* ATCC 25933, *Pseudomonas aeruginosa* ATCC 27853, *Salmonella typhimurium* ATCC 14028, *S. aureus* ATCC 6538P, *SStaphylococcus saprophyticus* ATCC 35552 and *Streptococcus agalactiae* ATCC 13813, and fungi: *Aspergillus flavus* ATCC 9170, *Aspergillus fumigatus* ATCC 26934, *Aspergillus niger* ATCC 9092, *Rhizopus* sp CL 35, *Microsporum canis* C112, *Microsporum gypseum* C115, *Trichophyton mentagrophytes* ATCC 9972, *Trichophyton rubrum* C137, *Cryptococcus neoformans* ATCC 32264, *Candida albicans* ATCC 10231 and *Candida krusei* ATCC 6582.

4.5.2. Media and inoculum

The bacteria used were cultivated on Mueller-Hinton agar (MHA – Difco) at 35 °C for 24 h. Cell suspension in saline (0.86%) was adjusted to give a final concentration of 1.5×10^8 cell/mL, standardized with 0.5 on the McFarland scale ($\lambda = 530$ nm) [24]. The fungi were cultivated on Sabouraud dextrose agar (SDA – Difco). For the filamentous fungi, suspensions were obtained according to the reported procedures, adjusted to between 1.0×10^6 and 5.0×10^6 spores/mL by microscopic enumeration with a cell counting hemocytometer (Neubauer chamber) [25], and the yeasts were prepared according to Pfaller et al. [26], adjusting the suspension to give a final concentration of between 1.0×10^6 and 5.0×10^6 cell/mL, also standardized with 0.5 on the McFarland scale ($\lambda = 530$ nm).

4.5.3. Quantitative antimicrobial evaluation

The minimum inhibitory concentration (MIC) was determined for the organisms by the agar dilution method, carried out on slants (1 mL). Stock solutions of each compound were diluted in dimethylsulfoxide (DMSO) to give serial two-fold dilutions that were added to each medium (MHA for bacteria and SDA for fungi), resulting in concentrations ranging from 10 to 100 μg/mL. Afterwards, a volume of 1 μL of inoculum suspension, prepared previously, was added to each slant, with the exception of the sterile control. The final concentration of DMSO in the assay did not exceed 2%. A drug-free solution was used as blank control and the antifungal agent ketoconazole (Sigma) was included in the assay as positive control. Each assay was repeated three times. The slants were incubated at 35 °C for the bacteria and yeasts and 28–30 °C for the hialohyphomycete and dermatophyte strains. MIC for each compound was defined as the lowest concentration that produces no visible microbial growth after the incubation time.

4.6. Cytotoxic activity

Artemia salina test was used for cytotoxicity determination of the compounds. Dried brine shrimp eggs (1 g/L) (Maramar, Arraial do Cabo, RJ, Brazil) were placed in artificial salt water consisting of 23 g NaCl, 11 g $MgCl_2 \cdot 6H_2O$, 4 g Na_2SO_4, 1.3 g $CaCl_2 \cdot 2H_2O$, 0.7 g KCl in 1000 mL distilled water. The pH was adjusted to 9.0 using Na_2CO_3 [27], and incubated at 25 °C with strong aeration, under a continuous light regime (2000 Lux).

Approximately 12 h after hatching, the phototropic nauplii were collected, and ten brine shrimp were transferred to each well using adequate pipettes. Ten brine shrimp were transferred to each well using pipettes. Each test consisted of exposing groups of 10 larvae aged 12 h to various concentrations of the compound. The toxicity was determined after 24 h of exposure. The numbers of survivors were counted and the percentage of deaths calculated. Larvae were considered dead if they did not exhibit any internal or external movement over several seconds of observation.

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