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Short communication

In vitro mutagenicity of anti-inflammatory parsalmide analogues PA7, PA10, and PA31 triggered by biotransformation into hydroxy derivatives

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Abstract

In this study, the mutagenicity of the anti-inflammatory parsalmide [5-amino-*N*-butyl-2-(2-propynyloxy)-benzamide] analogues PA7 [5-amino-*N*-butyl-2-cyclohexyloxy-benzamide], PA10 [5-amino-*N*-butyl-2-phenoxy-benzamide] and PA31 [5-amino-*N*-butyl-2-(*p*-tolyloxy)-benzamide] was determined by an Ames Salmonella assay. The experiments were performed by preincubating the compounds in the absence and presence of a post-mitochondrial fraction (S9) of rat liver homogenate from phenobarbital/β-naphtoflavone treated rats. No mutagenic effect was observed after direct testing (no S9 added) in *Salmonella typhymurium* strains TA98, TA100, TA102, TA1535 and TA1537. However, in the presence of S9, the test substances triggered mutagenic responses in strains TA100 and TA98. PA31 presented the strongest mutagenic potential. The reversion rates in the presence of PA31 were about 2–19 fold higher than spontaneous mutation rates. In the presence of PA7, the reversion increased 2–14-fold over spontaneous rates. While PA10 showed a relatively mild mutagenic potential, as the number of revertants did not exceed 2.5 times the number of spontaneous mutations. Mass spectrometric analysis of the in vitro biotransformation showed that S9 converted (%), regioselectively, PA7 (19%), PA10 (7%) and PA31 (12%) into hydroxy-derivatives.

Keywords: Parsalmide analogues; Mutagenicity; Metabolites; Mass spectrommetry

1. Introduction

Among the non-steroidal anti-inflammatory drugs (NSAIDs) that reached the public in recent decades, parsalmide [5-amino-*N*-butyl-2-(2-propynyloxy)-benzamide] (Fig. 1) is outstanding for causing no ulcerogenous nor hemorrhagic effects at the gastroduodenal level [1,2]. This desirable characteristic from the therapeutic point of view has therefore inspired studies on parsalmide analogues as new NSAIDs candidates [3].

Several preclinical studies are required in the process of developing new drugs in order to establish the real pharmacological properties and toxicity potential of the test substance. These include assays to predict mutagenicity, carcinogenicity and metabolism pathways since biotransformation can affect the drug safety and efficacy due to the formation of therapeutically active or toxic metabolites [4,5].

Several in vitro models for studying drug mutagenicity and metabolism have been proposed [6–9]. The bacterial reverse mutagenicity assay on *Salmonella typhimurium* known as the Ames test [10] is the most popular short term test for assessing the mutagenic potential of xenobiotics. The Ames test is the preferred screening method for the assessment of the mutagenic activity of new synthesized drugs. Some authors have assumed that there is a strong correlation between the carcinogenic activity in an animal experiment and the mutagenic potential in the Ames test [11]. However, more recently Fetterman et al. [12] established that the relationship between

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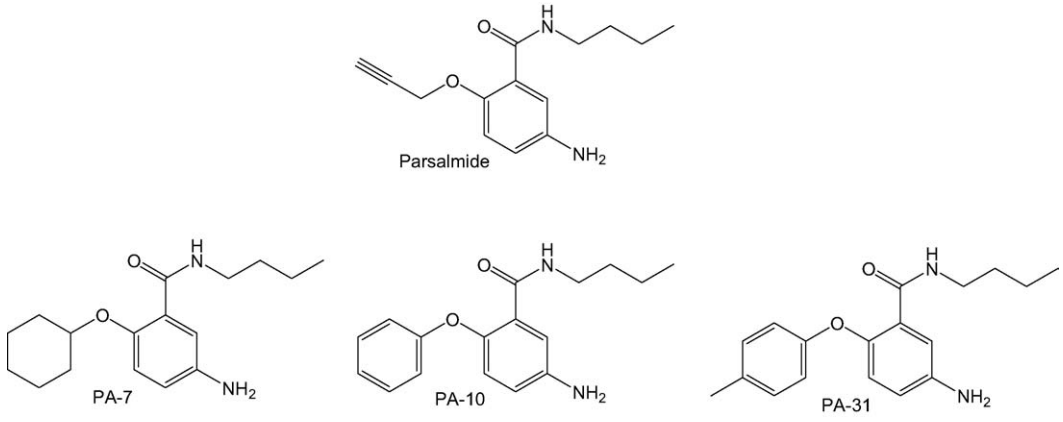


Fig. 1. Parsalmide and parsalmide analogues PA7, PA10 and PA31.

mutagenic potency predictors and quantitative carcinogenicity is very weak.

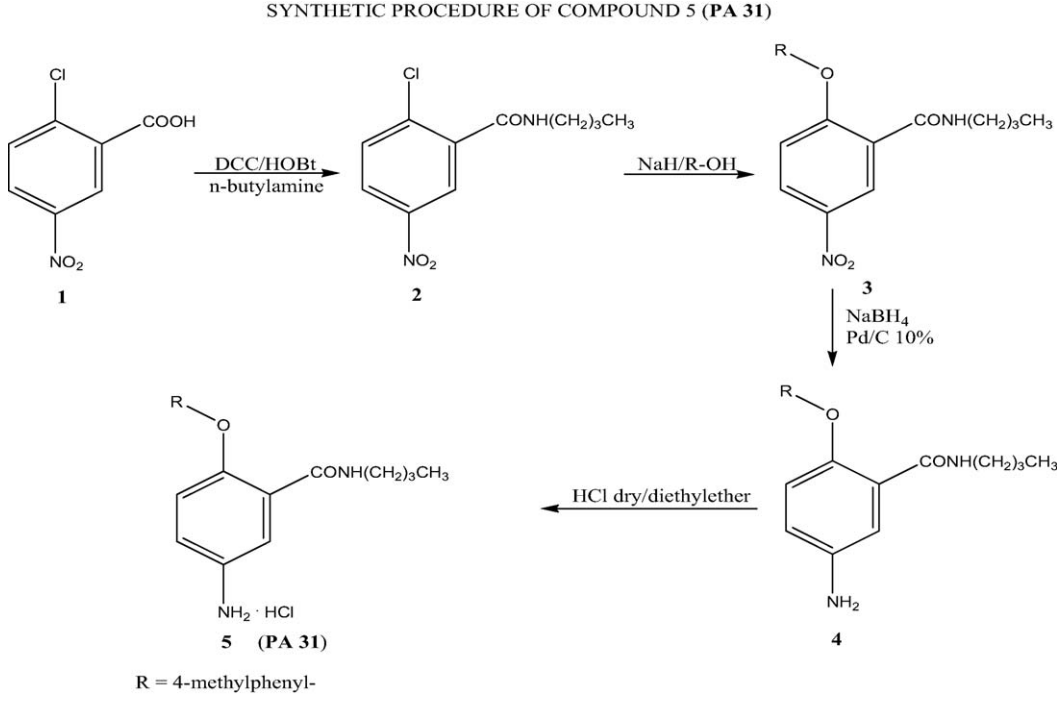
Liver preparations and isolated hepatocytes are routinely used for the prediction of in vitro metabolism of drug candidates with the obvious advantage that they are easier to work with than whole animals [13,14]. Liquid chromatography (LC) coupled with mass spectrometry (MS) is capable of generating rapid and accurate elucidation of the metabolites identity and plays a fundamental role in the development of new drugs [15–17].

A series of substituted benzamides has been synthesized as anti-inflammatory agents [3]. Two of the compounds, PA7 and PA10 presented very high anti-inflammatory activity in the carragenin-induced rat paw edema [3] and in vitro inhibition of human platelet aggregation (data not shown). Since both compounds were devoid of gastric effect at the efficacious dose and also prevented indomethacin-induced gastric damage, they were used to lead the definition of a new leading structure with anti-inflammatory activity. In a new series of compounds, PA31 was selected as presenting the highest anti-inflammatory activity in carragenin-induced paw edema and platelet COX-1 inhibition (data not shown). In order to further the understanding of this new class of anti-inflammatory drugs, the mutagenic potential of these compounds were evaluated by the Ames test and LC-MS analysis was applied for the purpose of characterizing related genotoxic metabolites formed in vitro in the presence of rat liver S9.

2. Chemistry

2.1. Synthesis of parsalmide analogue PA31

The general procedure used for the synthesis of the N-(n-butyl)-benzamide derivative PA31 is depicted in Scheme 1.



Scheme 1. Synthetic procedure of compound PA31.

The reaction of the 2-chloro-5-nitrobenzoic acid **1** with n-butylamine performed in anhydrous dimethylformamide (DMF) in the presence of N.N'- dicyclohexylcarbodiimide/1-hydroxybenzotriazole (DCC/HOBt) produced the corresponding intermediate **2**. Subsequent dissolution of the **2** in anhydrous dioxane and treatment with a solution of the appropriate alcohol and NaH in anhydrous dioxane gave the corresponding intermediate **3.** Reduction of the nitro group of intermediate **3**, carried out with sodium borohydride reagent, gave the corresponding intermediate **4.** Free base **4** was converted into the corresponding hydrochloride salt **5** by treatment with an excess of diethyl ether saturated with dry, gaseous HCl.

3. Results

3.1. Mutagenicity assay

Substances PA7, PA10 and PA31 failed to elicit a mutagenic response in the Ames test after direct testing (no S9mix) in strains TA98, TA100, TA102, TA1535 and TA1537. However, Tables 1a,1b show that in the presence of S9mix the test substances were converted into derivatives that induced at least a twofold increase in the number of revertant mutants of both TA98 and TA100. No increase in the revertant colonies was observed with strains TA102, TA1535 and TA1537. S9-activated PA7 and PA31 showed a dose-response effect in the dose range applied (31–250 μg/plate), although above 250 μg/plate, a general decrease was observed due to cell toxicity. Tables 1a,1b also show that S9-activated PA7 and PA31 presented distinct strain specific mutagenic tendencies. Whereas the greatest number of revertant mutants of TA98 in the presence of S9-activated PA7 was almost 15-fold higher than the spontaneous mutation rate, while in the presence of S9-activated PA31 the maximum increment was not higher than sevenfold. On the other hand, the number of revertants of TA100 in the presence of S9-activated PA7 was about fivefold the number of spontaneous mutations, whereas in the presence of S9-activated PA31 the revertion reached 19-fold the background level. In contrast, in the presence of S9-activated PA10 the number of reverted colonies did not exceed 2.5 times that of spontaneous mutations and no dose-response was observed.

3.2. MS characterization of PA metabolites

Fig. 2 shows the total ion chromatograms related to the LC-MS analysis of the in vitro incubations of PA7, PA10 and PA31 with S9mix. Starting materials were identified by comparison of the retention times and mass spectra with data obtained by direct injection of standard solutions, and the corresponding metabolites were characterized by interpretation of mass spectrometric data. As the atmospheric pressure ionization source is relatively soft, the positive electrospray full-scan spectra (m/z 150–350) of all compounds showed the pseudomolecular ions ($[M - H]^+$) in relatively high abundance (Fig. 3). Additionally, ions corresponding to sodium adducts ($[M - Na]^+$) were present in all spectra as the parent compound plus 23 mass units. The chromatographic peaks associated with metabolites of PA7, PA10 and PA31 eluted before the parent compounds, indicating that they were more hydrophilic products. The corresponding mass spectra showed an increment

Table 1a
Mutagenic activity of parsalmide analogues in the Ames test using the TA98 strain

Substance	Dose μg/plate	S9mix	Number of revertant colonies per plate			Mean number of revertants	S.D. (%)	Ratio test/control
DMSO		+	23	25	26	25	6	
PA7	1000	+	0	0	0	0	–	0.0[a]
	500	+	5	1	5	4	63	0.1[a]
	250	+	508	348	234	363	38	14.7
	125	+	260	246	244	250	3	10.1
	62.5	+	213	216	183	204	9	8.3
2AA	5	+	3080	4156	3350	3529	16	143.1
DMSO		+	23	25	26	25	6	
PA10	1000	+	0	0	6	2	173	0.0[a]
	500	+	31	5	2	13	126	0.5[a]
	250	+	40	25	15	27	47	1.1[b]
	125	+	57	64	60	60	6	2.4
	62.5	+	63	53	69	62	13	2.5
2AA	5	+	3080	4156	3380	3539	16	143.5
DMSO		+	32	25	17	25	30	
PA31	1000	+	143	123	114	127	12	5.1[b]
	500	+	186	172	169	176	5	7.1
	250	+	102	115	143	120	17	4.9
	125	+	111	128	109	116	9	4.7
	62.5	+	61	66	85	71	18	2.9
2AA	5	+	702	695	790	729	7	29.6

[a] Toxic: complete disappearance of background lawn.
[b] Moderately toxic: partial disappearance of background lawn.

Table 1b
Mutagenic activity of parsalmide analogues in the Ames test using the TA100 strain

Substance	Dose μg/plate	S9mix	Number revertant colonies per plate			Mean number of revertants	S.D. (%)	Ratio test/ control
DMSO		+	240	239	210	230	7	
PA7	500	+	666	757	673	699	7	3.0[a]
	250	+	754	875	788	806	8	3.5[a]
	125	+	1306	1244	1176	1242	5	5.4
	62.5	+	975	722	825	841	15	3.7
	31.25	+	566	615	481	554	12	2.4
2AA	5	+	3712	4118	4468	4099	9	17.8
DMSO		+	240	239	210	230	7	
PA10	500	+	537	573	470	527	10	2.3
	250	+	575	449	553	526	13	2.3
	125	+	560	501	547	536	6	2.3
	62.5	+	514	471	538	508	7	2.2
	31.25	+	479	604	458	514	15	2.2
2AA	5	+	4148	4468	3712	4109	9	17.9
DMSO		+	96	87	78	87.0	10	
PA31	500	+	1399	1539	1631	1523	8	17.5[a]
	250	+	1737	1757	1563	1686	6	19.4
	125	+	1132	1345	1331	1269	9	14.6
	62.5	+	1282	994	1157	1144	13	13.2
	31.25	+	608	623	674	635	5	7.3
2AA	5		1180	1379	1408	1322	9	15.2

[a] Moderately toxic: partial disappearance of background lawn.

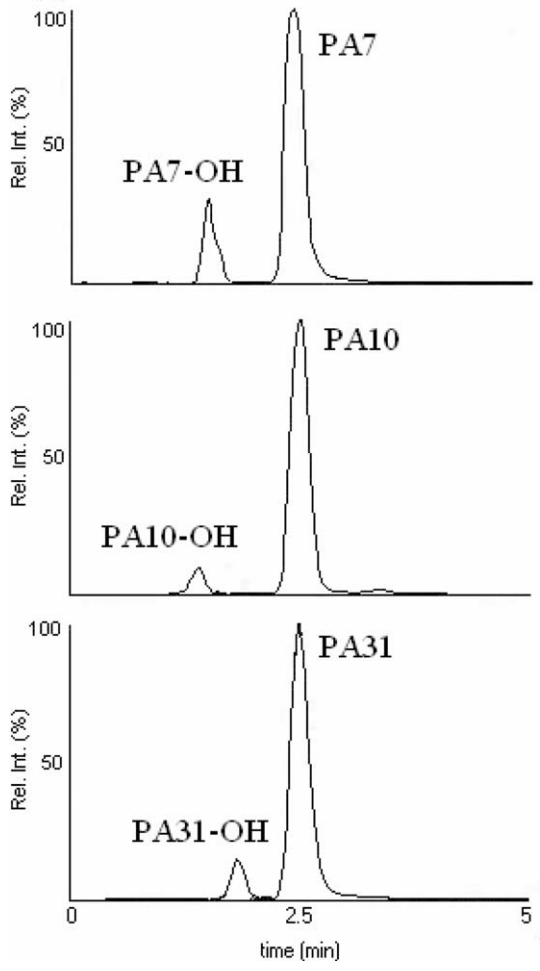


Fig. 2. Total ion chromatograms of ethyl ether extracts of Ames test-metabolic activation medium incubated with (A) PA7, (B) PA10 and (C) PA31 for 30 min.

of 16 mass units in both ($[M - H]^+$) and ($[M - Na]^+$), indicating that S9 converted PA7, PA10 and PA31 into hydroxy-derivatives. These were the only metabolites detected under the experimental conditions described. Percentual conversions (c%) by S9 of PA into hydroxy-products (PA-OH) depended on the substance structure, and in incubation conditions of a typical test dose of 31.25 μg/plate, c% of PA7, PA10 and PA31 were about 19%, 7%, and 12%, respectively (Table 2). Chemical groups subjected to hydroxylation were characterized by the full-scan mass spectra patterns shown in Fig. 3.

4. Discussion

Worldwide regulatory commissions considering in vitro mutagenicity assays have intensively debated the Ames test in terms of result evaluation, but still a consensus on the acceptable criteria for a positive or negative result could not be reached [20–23]. Nevertheless, it is widely believed that a reproducible dose-response is necessary for a chemical to be classified as positive and that the use of a specific two- or threefold increase rule is in fact too conservative for bacterial strains possessing high spontaneous mutation values yet not sufficiently conservative for those strains with low spontaneous mutation values [20].

S9-activated PA7 and PA31 clearly presented both concentration-related base-pair substitution (TA100) and frameshift (TA98) reversion effects. Biotransformation of primary arylamines into *N*-hydroxy mutagenic species has been largely demonstrated [24–26]. However the hydroxylation of PA7 most likely occurred in the cyclohexane ring, as the ions of *m/z* 209 in both PA7 and PA7-OH (Fig. 4) spectra were probably due to an inter-ring hydrogen rearrangement leading to neutral losses of cyclohexene and cyclohexenol, respectively. This interpreta-

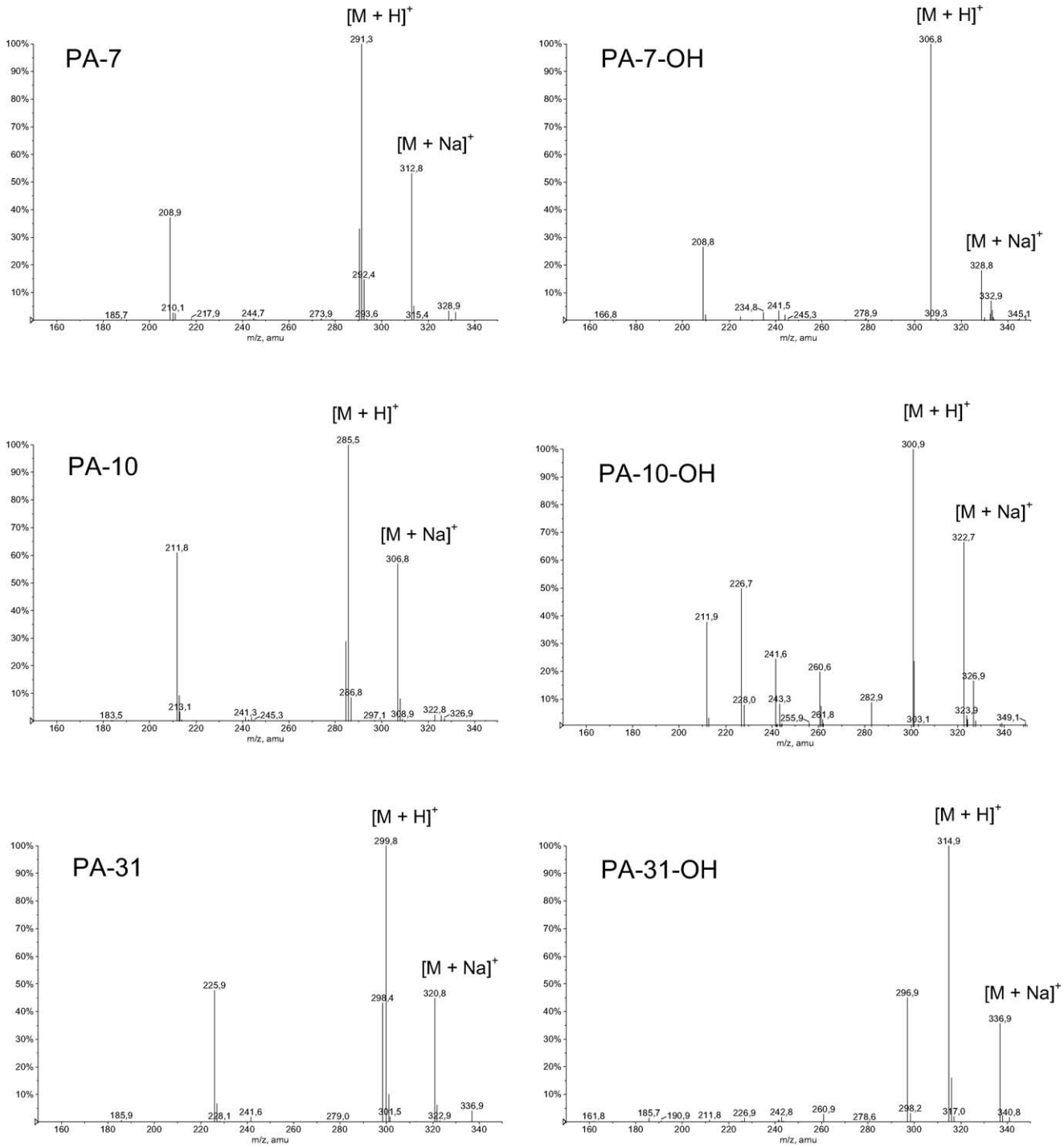


Fig. 3. ESI full-scan (*m*/*z* 150–350 Da) mass spectra of PA7, PA10, PA31 and corresponding S9-catalyzed hydroxy-derivatives.

tion is consistent with the fact that cyclohexane serves as substrate for several cytochrome P450 isoforms [27,28]. On the other hand, as shown in Fig. 5, the amine hydroxylation most likely occurred in the PA31 molecule, since in this case the positively charged mass fragment resulting from loss of H_2O (*m*/*z* 297) is relatively abundant (45%) and presented as a single product ion in the PA31-OH spectrum, probably due to an efficient *p*-oxygen-electron pair stabilization. It has been demonstrated that the enzymatic conjungation of arylamine *N*-oxidized metabolites can be of major concern due to their potential toxicity [25,29,30].

In regard to S9-activated PA10, no concentration-related increase in the number of revertant mutants was observed in the dose-range tested. Therefore, a clear mutagenic effect is not observed, but a discrete twofold increase in the number of revertant mutants relative to controls was obtained, indicating

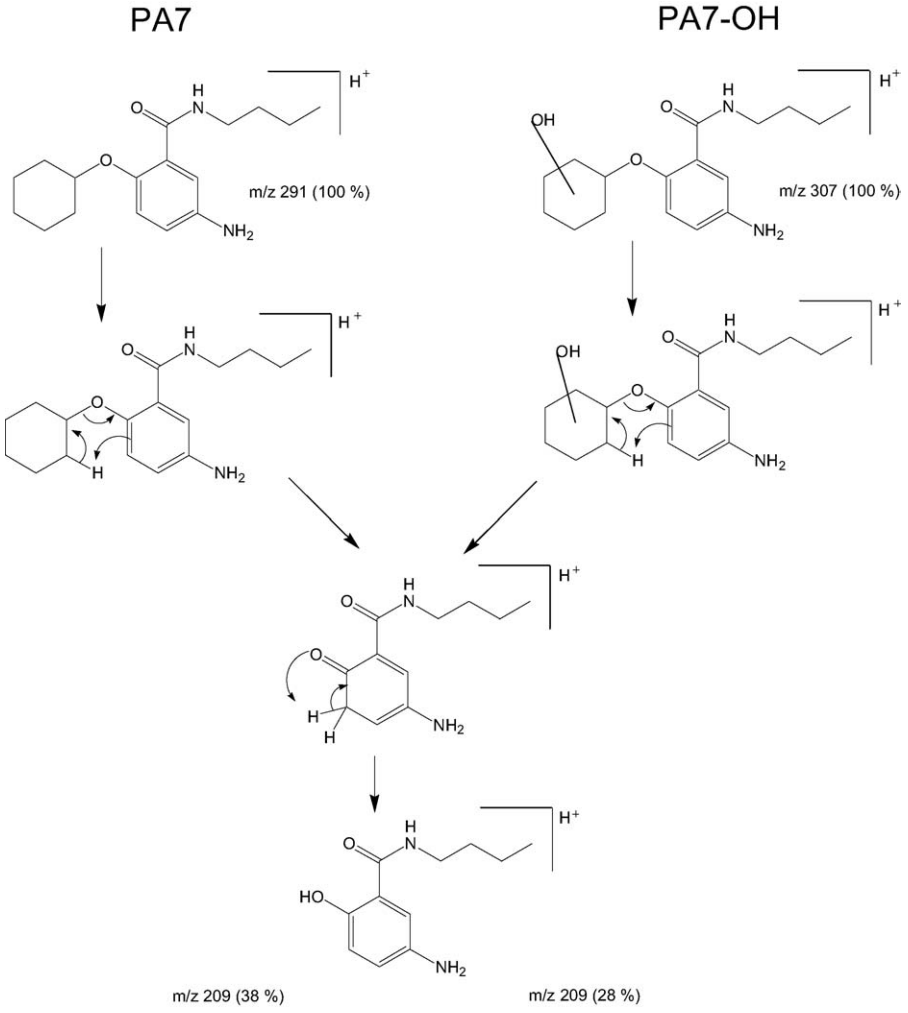


Fig. 4. Mass fragmentation mechanisms suggested for PA7 and PA7-OH.

Table 2
S9-mix conversion of parsalmide analogues into hydroxy-derivatives

Sample	dose/plate	t (min)[b]	TIC Area[a]		c%[c]
			PA	PA-OH	
PA-7	31.25	30	7.24×10^9	1.70×10^9	19
PA-10	31.25	30	8.91×10^9	6.84×10^8	7
PA-31	31.25	30	3.36×10^9	4.45×10^8	12

[a] Total ion chromatographic peak areas integrated as full scan spectra (m/z 150–350).
[b] Time (min) of incubation with S9-mix.
[c] Conversion calculated as $(M \times 100)/(A + M)$.

that mutagenic potential of PA10 needs further evaluation. Despite the PA10 and PA31 chemical resemblance, the mass spectrum analysis performed is in consistent with the biological assay results, since PA10-OH and PA31-OH showed completely different mass fragmentation patterns. In contrast to the primary amine hydroxylation of PA31, hydroxylation at the *N*-(*n*-butyl) amide moiety is shown for PA10-OH, as the ion of *m*/*z* 212 in both PA10 and PA10-OH (Fig. 6) spectra were most probably due to an acylium ion holding structural features fully shared by parent and product compounds. The efficiency of the Ames test for the S9-activated PA10 can be affected by the fact that the percent conversions (c%) by S9 of PA10 into hydroxy-product (PA10-OH) in a typical test dose of 31.2 μg/plate was about 7%, while PA7 and PA31 were about 19% and 12%, respectively.

In conclusion, our results show that liver S9-cytochrome P450 enzymes regioselectively convert the parsalmide analogues PA7, PA10 and PA31 into hydroxy-derivatives, which proved to be unsafe metabolites when evaluated for mutagenic potential in the Ames test. The study herein reported may guide the definition of a new leading structure with anti-inflammatory activity that may allow for the design of new safer NSAIDs.

5. Experimental protocols

5.1. Materials

The test substances PA7 and PA10 were synthesized as previously described [3] and supplied by Professor V. Santagada (Pharmaceutical Chemistry Department, University of Naples, Italy). All other chemicals were purchased from Sigma and

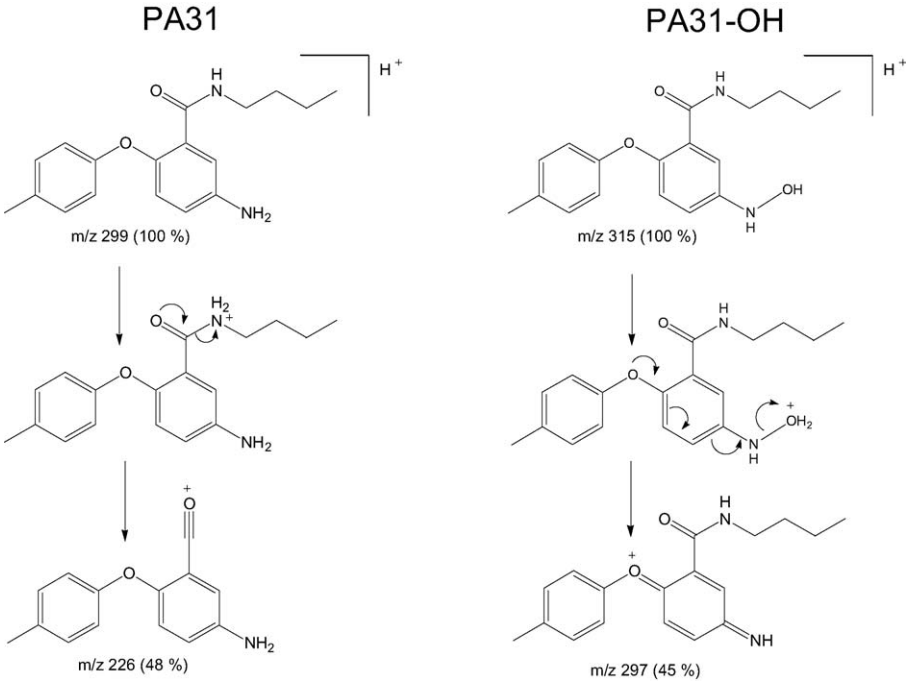


Fig. 5. Mass fragmentation mechanisms suggested for PA31 and PA31-OH.

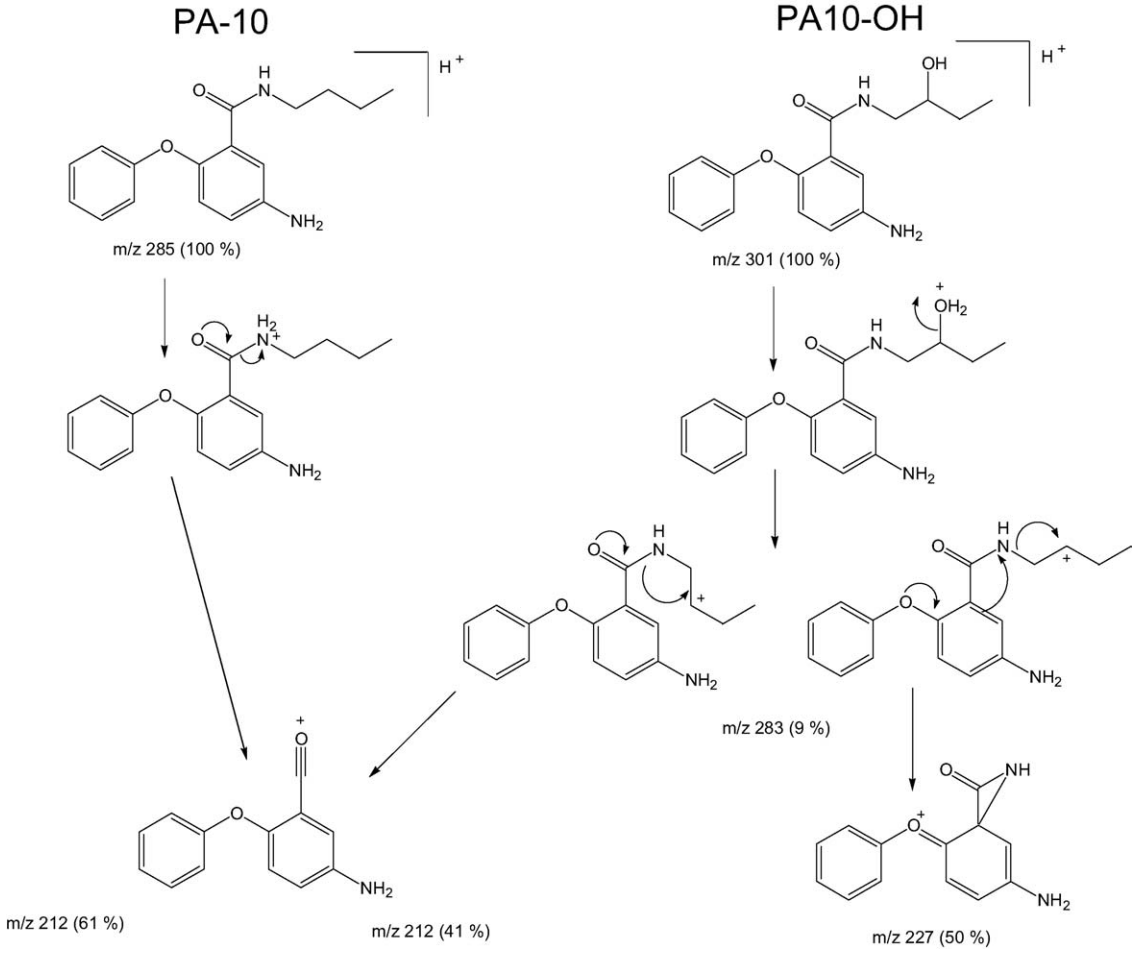


Fig. 6. Mass fragmentation mechanisms suggested for PA10 and PA10-OH.

were used without further purification. The strains of *Salmonella typhimurium* were purchased from Xenometrix (San Diego, CA, USA).

5.2. Chemistry

5.2.1. 5-amino-N-butyl-2-(p-tolyloxy)-benzamide (PA31)

1-hydroxybenzotriazole (HOBt) (14.7 g, 109 mmol) and dicyclohexylcarbodiimide (DCC) (22.5 g, 109 mmol) were added to a solution of 2-chloro-5-nitrobenzoic acid **1** (20 g, 99.0 mmol) in anhydrous DMF (150 ml) at 0 °C. The resulting reaction mixture was stirred for 30 min, and then n-butylamine (10.8 ml, 109 mmol) was added. The reaction mixture was stirred at 0 °C for 2 h and stored overnight at room temperature. *N*,*N*′-dicyclohexylurea (DCU) was filtered off, and the DMF was evaporated in a vacuum. The residue was dissolved in CH_2Cl_2, washed consecutively with brine, 1 N NaOH, brine, 1 N HCl and brine. The organic phase was dried over magnesium sulfate, filtered, concentrated in vacuum and, then the residue was purified by chromatography on a silica gel column (elution with diethyl ether/petroleum ether 7:3 v/v). Crystallization from diethyl ether/ethanol produced 19.6 g (77%) of pure **2** as a yellow solid, m.p. 134 °C. ^{1}H-NMR ($CDCl_3$) δ 8.46 (d, 1H, $J = 2.9$ Hz, ArH), 8.19 (dd, 1H, $J = 2.9$ Hz, ArH), 7.59 (d, 1H, $J = 7.9$ Hz, ArH), 6.23 (br, 1H, CONH), 3.48 (q, 2H, $J = 6.9$ Hz, CH_2NH), 1.89 (q, 2H, $J = 6.9$ Hz, CH_2CH_2NH), 1.44 (m, 2H, $J = 7.9$ Hz, CH_2CH_3), 0.98 ppm (t, 3H, $J = 6.9$ Hz, CH_2CH_3). Next, a solution of sodium hydride (60% in mineral oil, 1.87 g, 78.0 mmol) in dry dioxane (100 ml) was added to p-cresol (5.57 g, 51.0 mmol) in an ice-water bath under a nitrogen atmosphere. After the evolution of hydrogen had ceased, the mixture was stirred at 70 °C for 2 h. Then intermediate **2** (10.0 g, 39.0 mmol) in anhydrous dioxane (80 ml) was added drop-wise, and the resulting reaction mixture was stirred at 70 °C for 4 h and then overnight at ambient temperature. The solvent was removed under reduced pressure and the residue was dissolved in ethyl acetate and washed with brine. The organic phase was dried over magnesium sulfate, filtered, concentrated in vacuum and the residue purified by chromatography on a silica gel column (elution with hexane/ethylacetate 8:2 v/v) to produce intermediate **3** (15 g, 89%), m. p. 77 °C. ^{1}H-NMR ($CDCl_3$) δ 9.12 (d, 1H, $J = 2.9$ Hz, ArH), 8.15–8.13(dd, 1H, $J = 2.9$ Hz, ArH), 7.69 (br, 1H, CONH), 7.29 (d, 2H, $J = 7.9$ Hz, ArH), 7.02 (d, 2H, $J = 7.9$ Hz, ArH), 6.82 (d, 2H, $J = 7.9$ Hz, ArH), 3.51 (q, 2H, $J = 6.9$ Hz, CH_2NH), 2.40 (s, 3H, CH_3), 1.61 (q, 2H, $J = 6.9$ Hz, CH_2CH_2NH), 1.41 (m, 2H, $J = 7.9$ Hz, CH_2CH_3), 0.93 ppm (t, 3H, $J = 6.9$ Hz, CH_2CH_3), which was converted to **4** as follows: A suspension of (50 ml) of $NaBH_4$ 3.65 g (96.5 mmol) in water was added to a suspension of Pd/C (0.59 g) in 40 ml of water. The resulting reaction mixture was stirred at room temperature under nitrogen for 10 min. Then **3** (15.82 g, 48.2 mmol) in 150 ml of methanol was added drop-wise, and the mixture was stirred for 30 min. The reaction mixture was filtered through a Celite. The solution was acidified with 1N HCl to remove the excess of $NaBH_4$. The acidified solution was adjusted to alkaline pH with 2N NaOH solution and the resulting suspension was extracted with diethyl ether. The organic phase was dried over magnesium sulfate, filtered, then evaporated to dryness to yield intermediate **4** as a brown oil (13.7 g, 95%); ^{1}H-NMR data are reported as hydrochloride salt. Free base **4** (13 g) was dissolved in ethanol (100 ml), treated with an excess of diethyl ether saturated with dry, gaseous HCl. Recrystallization from diethyl ether/ethanol (8:2, v/v) provided **5** (13g, 89%) as a white solid; ^{1}H-NMR ($CDCl_3$) δ 8.36(s, 1H, CONH), 7.81(d,1H, $J = 2.9$ Hz, ArH), 7.72 (d, 1H, $J = 7.9$ Hz, ArH), 7.25 (d, 2H, $J = 7.9$ Hz, ArH), 6.92 (d, 2H, $J = 7.9$ Hz, ArH), 6.80 (d, 1H, $J = 2.9$ Hz, ArH), 3.39 (q, 2H, $J = 6.9$ Hz, CH_2NH), 2.34 (s, 3H, CH_3), 1.50 (q, 2H, $J = 6.9$ Hz, CH_2CH_2NH), 1.31 (m, 2H, $J = 6.9$ Hz, CH_2CH_3), 0.85 ppm (t, 3H, $J = 6.9$ Hz, CH_2CH_3). MS *m*/*z* 299.2 (M^+).

5.3. Instrumentation

Absorbance measurements were performed on a Shimadzu model 1240 UV-Visible spectrophotometer. The HPLC system consisted of two Shimadzu LC10AD solvent pumps and a CTC Analytics PAL System autosampler. Mass spectra were obtained using Applied Biosystems API 4000 electrospray ionization (ESI) spectrometer.

5.4. Mutagenicity assay (Ames test)

Mutagenicity was assessed in the Ames Salmonella assay with strains TA98, TA100, TA102, TA1535 and TA1537. Recipes for reagents and media were performed as described by Mortelmans and Zeiger [18]. Bacteria were grown in nutrient broth (25 g/l, #2 Oxoid) for 15 h, at 37 °C, and 120 rpm, to give suspensions of 3×10^8 cells/ml ($A_{550nm} = 0.25$; McFarland scale, bioMérieux, Lyon, France). Test compounds were dissolved in dimethylsulfoxide (DMSO) to give solutions of 50, 25, 12.5, 6.2, 3.1 and 1.55 mg/ml. Assays without metabolic activation (no S9) were performed mixing 20 μl of each test substance solution with 500 μl of phosphate buffer (0.1 M, pH 7.4) and 100 μl of bacteria suspension. After 30 min of incubation, 2 ml of molten top agar supplemented with traces of histine and biotine (50 μM each, final concentration) were added, rapidly vortexed and poured on GM agar plates. As the top agar hardened, plates were inverted and incubated for 48 h, at 37 °C. Assays with in vitro metabolism were similarly performed replacing phosphate buffer by an equal volume of S9mix (10% v/v S9, 4.7 mM NADP, 6 mM D-glucose-6-phosphate, 19 mM $MgCl_2$, 36 mM KCl, phosphate buffer 0.1 M pH 7.4). Rat liver S9 was prepared as described by Ames et al. [19], except that rats were treated for 3 consecutive days with intraperitoneal injections of phenobarbital (100 mg/kg) and β-naphthoflavona (80 mg/kg). Positive controls were performed with strain-specific substances largely used in the Ames assay [18]. Negative controls were performed with plain DMSO. Experiments were run in triplicate. The results were

recorded as mean revertant colonies per plate ± the (%)S.D. and as the ratio (R) of the number of revertant colonies per test plate and negative control. A positive response for mutagenicity was defined as a reproducible twofold increase of revertants with a dose–response relationship [20–23].

5.5. In vitro biotransformation

Reactions were performed based on the mutagenicity assay procedure described above. Test compounds were dissolved in DMSO to give solutions of 3, 1.5 and 0.3 mg/ml. Aliquots (20 μl) from these were added to bacteria suspensions (100 μl, 3×10^8 cells/ml) and S9mix (500 μl). The mixtures were incubated at room temperature (25 °C) for 30 min, and then extracted with 3 ml of ethyl ether (vortex, 60 s). The organic phases were separated, solvents evaporated under N_2 and final residues suspended in CH_3CN–H_2O/0.1% formic acid (1:1) for LC-MS analysis. Control reactions were performed using a cofactor mix devoid of S9.

5.6. HPLC-MS analysis

Chromatographic separations were carried out on a Genesis C8 column (120 × 4.6 mm I.D., 4 μ particle size, Genesis, UK). C8 guard column (4 × 4 mm I.D., Genesis) was used to protect the analytical column. The mobile phase for the analysis of the test substances and their metabolites consisted of CH_3CN-H_2O (45:55, v/v) acidified with 0.1% formic acid, at a flow rate of 1 ml/min. The autosampler was maintained at 8 °C and was set up to make a 10 μl sample injection. The MS system was operated with the ESI interface in the positive ion mode in the 150–350 Da range. The source block temperature was 450 °C. The ionization energy was 4500 V. Nitrogen was used as drying, nebulizing and collision gases. Conversion rate (c%) was calculated as $(M \times 100)/(A + M)$, where A = chromatographic peak area of the starting material and M = chromatographic peak area of the metabolite.

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