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The synthesis, characterization and biological evaluation of a new nitric oxide donor agent

LENUTA PROFIRE[1]*, MARIA APOTROSOAEI[1], ANCA OPREA[2], MIHAI BREBU[2], FLORENTINA LUPASCU[1], CATALINA ELENA LUPUSORU[3] and CORNELIA VASILE[2]

[1]*Department of Pharmaceutical Chemistry, Faculty of Pharmacy, "Grigore T. Popa" University of Medicine and Pharmacy, 16 University Str., 700115, Iasi, Romania,* [2]*Department of Physical Chemistry of Polymers, "P. Poni" Institute of Macromolecular Chemistry, Romanian Academy, 41A Grigore Ghica Voda Alley, 700487, Iasi, Romania and* [3]*Department of Pharmacology, Faculty of Medicine, "Grigore T. Popa" University of Medicine and Pharmacy, 16 University Str., 700115, Iasi, Romania*

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Abstract: The synthesis of a new xanthine nitric oxide donor (TSP-81) is discussed. The designed compound included two structural moieties, *i.e.*, theophylline (1,3-dimethylxanthine) and acetaminophen (4-hydroxyacetanilide), linked by the nitric oxide donor alkyl chain as a spacer. The compound was characterized by microanalysis (CHN), ^{1}H-NMR, ^{13}C-NMR, FT-IR and UV–Vis spectroscopy and thermogravimetric analysis. The thermal behaviour showed that TSP-81 melts with decomposition in four steps, the most important ones being the 2nd one (the registered weight loss being 17.6 %) and the 3rd one (with a registered weight loss of 30.4 %). The toxicity degree, the anti-inflammatory effect and the ability of releasing nitric oxide of TSP-81 was also evaluated. The biological assays established that TSP-81 exhibits enhanced biological properties, such as lower toxicity and higher anti-inflammatory effect, compared to theophylline and acetaminophen, the drugs used as the parent molecules. Thus, TSP-81 is approximately 2 times more active than theophylline and 4 times more active than acetaminophen in reducing cotton pellet granuloma formation. Furthermore, the release of nitric oxide (NO) appears to play an important role in enhancing the anti-inflammatory effect.

Keywords: xanthine; acethaminophen; toxicity; anti-inflammatory.

INTRODUCTION

In the last few years many research groups have focused their efforts on the discovery and development of "molecular hybrids" characterized by a nitric oxide (NO) releasing moiety, in order to enhance the pharmacological profile of

* Corresponding author. E-mail: nprofire@yahoo.com
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the parent drug.[1] NO is an important endogenous mediator generated by nitric oxide synthase (NOS) enzymes.[2] NO, biosynthesised by endothelial cells, is mainly considered to be a fundamental modulator of cardiovascular function, where it acts as a powerful vasodilator, inhibits platelets activation/aggregation and is involved in ischemic preconditioning, thus ensuring important cardioprotective effects.[3] In 1998, R. Furchgott, L. Ignarro and F. Murad were awarded the Nobel Prize in Physiology and Medicine for their discoveries concerning NO as a signalling molecule in the cardiovascular system. Increasing data suggest a considerable "modulating" effect of nitric oxide as a pleiotropic agent, acting on several body parts.[4–6] There is important evidence that NO is involved in several inflammatory disorders. Indeed, every cell and many immunological parameters are virtually modulated by NO.[7] It has been shown that NO can be pro-inflammatory (immunostimulatory, anti-apoptotic) or anti-inflammatory (immunosuppressive, pro-apoptotic), host-protective or host-damaging during infections.[8] For these reasons, NO has been described as a "double edge sword mediator" and this phenomenon is often referred to as the NO paradox. It is interesting to note that NO–NSAIDs exert analgesic and anti-inflammatory effects more potently than their parent NSAIDs (non-steroidal anti-inflammatory drugs), while they produce comparable suppression of prostaglandin (PG) synthesis.[7] The most suitable explanation for this is that the nitric oxide released by NO–NSAIDSs contributes to their analgesic and anti-inflammatory effects. The evidence to support this statement comes from the studies of an NO-releasing derivative of acetaminophen. It is known that acetaminophen is not a cyclooxygenase (COX) inhibitor, and exerts little if any anti-inflammatory effects.[9,10] However, an NO-releasing derivative of acetaminophen (NCX-701) was found to reduce carrageenan-induced paw oedema.[10]

Xanthines have been recognized for a long time for their use in the treatment of airways diseases, largely from the standpoint of the ability of these drugs to elicit bronchodilatation.[11] Theophylline has been used in the treatment of obstructive lung diseases, such as asthma and chronic obstructive pulmonary disease (COPD), for more than 75 years.[12] The molecular mechanism of bronchodilatation is likely explained by inhibition of phosphodiesterase (PDE) isoenzymes (III, IV and V). Recently, it has become clear that a number of inflammatory cells specifically possess the PDE isoenzyme IV, thus enhancing the chances that modulation of this enzyme would lead to anti-inflammatory effects. In support of this hypothesis, it has become increasingly apparent that xanthines, such as theophylline, and more selective PDE inhibitors, possess anti-inflammatory and immunomodulatory actions that may contribute to their clinical effects.[12]

In previous papers, the syntheses of new theophylline derivatives[13–15] and nitric oxide donors[16] were reported. In continuation of these studies, herein the synthesis, characterization and biological evaluation of a new xanthine nitric

oxide donor (TSP-81), as a potential drug useful in the treatment of asthma and chronic obstructive pulmonary disease, are presented.

EXPERIMENTAL

All chemicals were purchased from commercial sources (Sigma–Aldrich, Fluka) and were used without further purification. Solvents were purified and dried by standard methods. Melting points were determined by the open capillary method using the Buchi M 565 instrument. The progress of the reaction was monitored by thin-layer chromatography (TLC). The analysis of the CHN contents was realized on a Perkin Elmer 2400 II elemental analyzer. The UV–Vis spectra were recorded in an ethanol: water mixture (9:1) using an HP 8450A UV–Vis spectrophotometer. The FT-IR spectra were taken using the KBr pellet technique on a Bruker VERTEX 70 (USA) instrument, over the 500–4000 cm^{-1} wavenumber range, at a resolution of 4 cm^{-1}. The ^{1}H-NMR and ^{13}C-NMR spectra were recorded employing a Bruker Avance DRX-400 spectrometer using tetramethylsilane as an internal standard and DMSO-d_6 as solvent. Mass spectra were recorded using Agilent 6410 triple quadrupole mass spectrometer. The TG/DTG curves were recorded on a Paulik–Paulik–Erdey derivatograph under the following operational conditions: heating rate of 10 °C min^{-1}, temperature range of 25–600 °C, sample weight of ≈20 mg, platinum crucible and an air flow rate of 100 cm^3 min^{-1}. For each TG stage, the following thermal characteristics were determined: onset temperature (T_i), temperature corresponding to the maximum weight loss rate (T_m), and the temperature corresponding to the end of stage (T_f) (the errors in the temperature determinations were ±2 °C), the weight loss errors were ±1 % and the errors in the activation energy determinations were from ±15 to ±20 kJ mol^{-1}.

*Synthesis of 7-[3-(4-(acetylamino)phenoxy)-2-hydroxypropyl]-1,3-dimethyl-8-(morpholin-4-yl)-xanthine (**5**)*

7-[3-(4-(Acetylamino)phenoxy)-2-hydroxypropyl]-1,3-dimethyl-8-(morpholin-4-yl)xanthine (**5**) was prepared according to a literature procedure by reaction of 8-(morpholin-4-yl)--1,3-dimethylxanthine (**3**) with 4-(2,3-epoxypropoxy)acetanilide (**4**).[13] Yield: 68.6 %.

*Synthesis of 7-[-3-(4-(acetylamino)phenoxy)-2-((2-chloroacetyl)oxy)propyl]-1,3-dimethyl-8-(morpholin-4-yl)xanthine (**6**)*

7-[3-(4-(Acetylamino)phenoxy)-2-hydroxypropyl]-1,3-dimethyl-8-(morpholin-4-yl)xanthine (**5**, D6) (6.14 g, 13 mmol) in tetrahydrofuran (THF) (150 mL) was reacted with chloroacetyl chloride (1.12 mL, 14 mmol) and then triethylamine (TEA, 3.9 mL, 28 mmol) was added. The mixture of reaction was stirred for 10 h, at room temperature and then the solvent was distilled off under reduced pressure. Subsequently, the solid was washed with water, filtered and dried under vacuum. The product was recrystallized from absolute ethanol. Yield: 74.6 %.

*Synthesis of 7-[-3-(4-(acetylamino)phenoxy)-2-((2-(nitrooxy)acetyl)oxy)propyl]-1,3-dimethyl--8-(morpholin-4-yl)xanthine (**7**, TSP-81)*

To a solution of 7-[-3-(4-(acetylamino)phenoxy)-2-((2-chloroacetyl)oxy)propyl]-1,3--dimethyl-8-(morpholin-4-yl)xanthine (**6**) (5.49 g, 10 mmol) in dimethylformamide (DMFA, 150 mL) was added silver nitrate (1.87 g, 11 mmol) in DMFA (50 mL). The reaction mixture was stirred at room temperature for 24 h, in the dark and then filtered under vacuum. Afterwards, the liquid was distilled off under reduced pressure and the solid was washed with cold diethyl ether. Finally, the precipitate was filtered and dried under vacuum. The product was recrystallized from absolute ethanol. Yield: 63.5 %.

Acute toxicity assay

The acute toxicity was evaluated with the lethal dose test involving the administration of the compounds at increasing doses in order to determine the dose that would kill 50 % of the mice (LD_{50}) within a set time-frame.[17,18] The Swiss albino mice of either sex, aged 6 to 8 weeks weighing 20 to 25 g, were obtained from the Central Animal House, University of Medicine and Pharmacy "Grigore T. Popa", Iasi, Romania. The animals were kept in polyethylene boxes, in a controlled environment – constant temperature (24±2 °C) with a 12 h light–dark cycle and relative humidity of 40–70 %. They were kept without food for 24 h before the experiment and water was *ad libitum*. Groups of six mice were used and the studies were performed in accordance with the current guidelines for the veterinary care of laboratory animals,[19] and under the consent of the Ethics Committee for Animal Research of "Grigore T. Popa" University of Medicine and Pharmacy, Iasi, Romania. Each group was treated intraperitoneally with different concentrations of compounds in 0.5 % sodium carboxymethylcellulose (25, 50 and 75 mg mL^{-1}). A group of animals treated with 0.5 % carboxymethylcellulose was used as control group. The symptoms of toxicity and mortality were observed after 24, 48 and 72 h after administration and the LD_{50} of the compounds was estimated.

Cotton pellet-induced granulation inflammation

Tissue granulation was induced by surgical subcutaneous implantation of two cotton pellets in the dorsal region of the rats according to a published procedure[20] with a few modifications. Male Wistar rats, weighing 200–250 g, were divided into five groups of six animals each. Cotton pellets (60±2 mg each) were sterilized by dry heat at 160 °C for 2 h and aseptically implanted in the interscapular distance under the skin of the previously shaved back of the rats, which were anesthetized with thiopental sodium (25 mg kg^{-1}, *i.p.*). The compounds were administered orally at a concentration of 1/5 of the LD_{50} as a suspension in 0.5 % sodium carboxymethylcellulose (40 mg mL^{-1}) once a day for a period of six days. The animals of the control group received only the vehicle. On the seventh day, the rats were sacrificed and the pellets covered with the granulation tissue were extracted and dried in a hot air oven at 60 °C overnight. The difference between the final and initial weight of the pellets was regarded as the granuloma tissue production. A comparison was made between the granulation weight of the treated and control groups.

Detection of nitrite

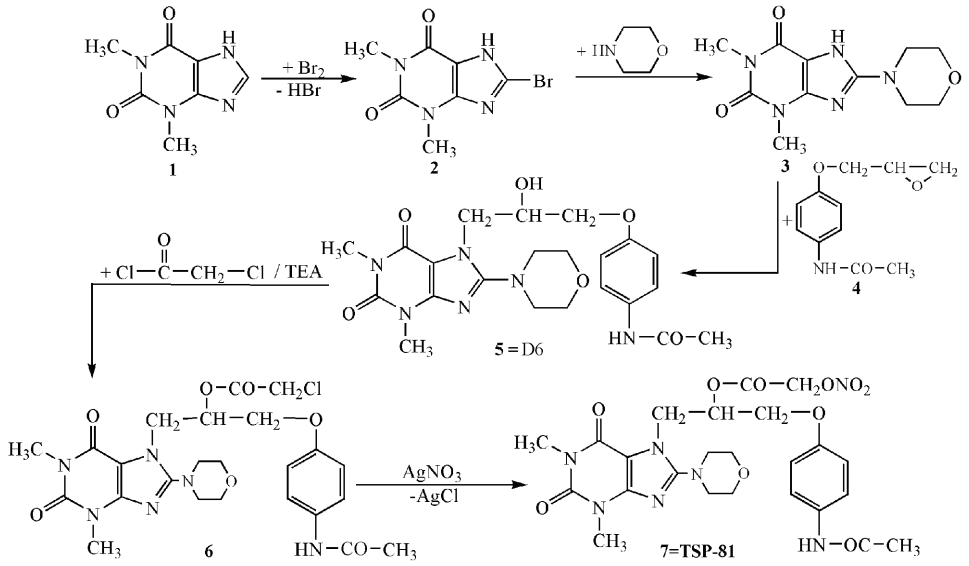
Different volumes (0.05, 0.1 and 0.2 mL) of the compound (TSP-81) in dimethyl sulfoxide (DMSO) were added to 2 mL of a mixture of 50 mM phosphate buffer (pH 7.4) with methanol solution containing 5×10^{-4} M cysteine (1:1, *V*/*V*). After 1 h at 37 °C, 1 mL of the reaction mixture was treated with 1 mL of Griess reagent (sulphanilamide (4 g), *N*-(1--naphthyl)-ethylenediamine dihydrochloride (0.2 g), 85 % phosphoric acid (10 mL) in distilled water (final volume 100 mL)).[21] After 10 min at room temperature, the absorbance was measured at 540 nm. Sodium nitrite standard solutions (10–80 nmol) were used for the calibration curve. The results are expressed as the percentage of the NO release (n = 3) to a theoretical maximum release of 1 mol NO mol^{-1} of the test compound.

Statistical analysis

The statistical significance of the results was analyzed by the student's *t*-test. The results are expressed as the mean ± standard error of mean (*SEM*). Values of $P \leq 0.05$ were considered statistically significant.

RESULTS AND DISCUSSION

As shown in Scheme 1, 7-[-3-(4-(acetylamino)phenoxy)-2-((2-(nitrooxy)-acetyl)oxy)propyl]-1,3-dimethyl-8-(morpholin-4-yl)xanthine (TSP-81) was synthesized in several steps. In the first step, theophylline (1,3-dimethylxanthine) (**1**) by reaction with bromine in glacial acetic acid afforded 8-bromotheophylline (**2**), which was then treated with morpholine in ethanol solution, when 1,3-dimethyl--8-(morpholin-4-yl)xanthine (**3**) was obtained. In the next step, the intermediary **3** on reaction with 4-(2,3-epoxypropoxy)acetanilide (**4**) led to 7-[3-(4-(acetylamino)phenoxy)-2-hydroxypropyl]-1,3-dimethyl-8-(morpholin-4-yl)xanthine (**5** = = D6).[13] Subsequently, **5** was reacted with chloracetyl chloride in tetrahydrofuran in the presence of triethylamine as acceptor of protons to give the chloroacetyl derivative (**6**) was obtained. Finally, **6** on reaction with silver nitrate in dimethylformamide afforded 7-[-3-(4-(acetylamino)phenoxy)-2-((2-(nitrooxy)-acetyl)oxy)propyl]-1,3-dimethyl-8-(morpholin-4-yl)xanthine (**7** = TSP-81).



Scheme 1. Synthesis of TSP-81.

The structures of the new derivatives were proved by elemental analyses, UV–Vis, IR, ^{1}H-NMR, ^{13}C-NMR and mass spectroscopy.

The physical, analyticcal and spectral data of **5**–**7** are given in the Supplementary material to this paper.

The IR spectra of the compounds showed peaks for xanthine, acetaminophen and the alkyl chain, which confirm the structure of the compounds. The strong absorption bands at 1740 and 1755 cm^{-1} are due to the ester CO stretching vibration of the chloroacetyl intermediary (**6**) and TSP-81, respectively. The alkyl

halide bond (**6**) appeared at 690 cm^{-1} as a strong band that was absent from the spectrum of TSP-81. In the spectrum of TSP-81, a strong band at 1314 cm^{-1} due to the stretching vibration of the $–ONO_2$ group was observed.

The ^{1}H-NMR spectra (DMSO-d_6) indicated the presence of protons from the alkyl chain that linked the xanthine structure with the acetaminophen residue. The protons of the three methylene groups of TSP-81 appeared as two doublets at 3.91 (CH_2–C) and 4.12 (CH_2–O) and as singlet at 4.41 ppm (CH_2–CO). The proton of the methine group (CH) appears as a singlet at 4.52 ppm. In the ^{13}C-NMR spectra of the compounds **6** and TSP-81, the signals of the alkyl chain carbons appeared at 41.23, 74.49, 75.34 and 172.47 (**6**) and at 40.84, 74.75, 75.21, 169.52 (TSP-81). In the pectrum of the chloroacetyl derivative, the signal for the carbon that linked halide was identified at 50.57, while the carbon that linked the nitrate ester group (TSP-81) appeared at 68.81.

The results of the elemental analysis and mass spectroscopy were found to be in agreement with the values that were theoretically calculated.

Thermogravimetic analysis (TG/DTG) was used to describe the thermal behaviour of the derivatives. The thermogravimetic analysis of TSP-81 showed its four-step decomposition. The first step was insignificant as it showed a mass loss of only 4 %, probably resulting from traces of solvent or water remaining in the compound. The next two overlapping steps were the main steps of the decomposition process that started at a temperature of 167 °C. These two steps accounted for 17.6 and 30.4 % of the mass loss, respectively. The maximum rate of mass loss corresponding to these two steps occurred at 295 and 345 °C, respectively. The last thermogravimetric step started at 462 °C and ended at 725 °C with the maximum rate of mass loss occurring at 615 °C. This step corresponded to the decomposition of an intermediary compound (D6), which left a residue of 16 %. The thermogravimetric characteristics (T_i, T_m, T_f and mass loss) are summarized in Table I.

TABLE I. Characteristic data of thermogravimetric steps; T_i – the onset temperature; T_m – the temperature corresponding to the maximum rate of mass loss; T_f – the temperature corresponding to the end of a decomposition stage

Compound	Step	T_i / °C	T_m / °C	T_f / °C	Mass loss, %
TSP-81	1	60	112	167	4.0
	2	167	295	313	17.6
	3	313	345	462	30.4
	4	462	615	725	32.0
D6	2 and 3	244	300	473	5.4
			337		45.7
			389		57.8

The kinetic parameters of the thermal decomposition (activation energy, E_a, and reaction order, n), were evaluated using both integral and differential methods

employing a versatile commercial programme that gives the overall kinetic parameters by means of various methods. Four methods, *i.e.*, the Coats–Redfern, the Flynn–Wall, the van Krevelen and the Urbanovici–Segal methods, were used to evaluate the overall kinetic parameters.[22,23] The global kinetic parameters for each thermogravimetric step are given in Table II, the results obtained using the different methods being equivalent.

TABLE II. The global values of the kinetic parameters for thermal decomposition of the TSP-81

Kinetic parameter	Step 2	Step 3	Step 4	Kinetic parameter	Step 2	Step 3	Step 4
Coats–Redfern				Flynn–Wall			
E_a / kJ mol^{-1}	37.2	195.6	103.7	E_a / kJ mol^{-1}	43.4	196.3	112.1
n	0.0	2.9	1.2	n	0.0	2.9	1.2
van Krevelen				Urbanovici–Segal			
E_a / kJ mol^{-1}	47.5	232.8	137.4	E_a / kJ mol^{-1}	39.7	200.8	107.1
n	1.4	3.0	1.4	n	0.0	3.0	1.2

As expected, the global activation energy (E_a) increased with the temperature of the decomposition. The first step occurred by a zero reaction order mechanism, being diffusion controlled. For the other two steps, the reaction order increased, showing that the mechanism of decomposition was very complex. The decrease of the activation energy for the four steps could be due to the formation of an unstable intermediary at higher temperatures.

Comparing all the thermo-oxidative characteristics, it appears that the NO--donor compound (TSP-81) is less thermally stable than the intermediary D6 xanthine derivative. It could be supposed that the nitrooxyacetyl chain that links theophylline to acetaminophen destabilizes the molecule because of the increasing oxygen content that allows for an easier oxidation.

Acute toxicity assay

The LD_{50} (the dose causing the death of 50 % of the tested animals) is usually the initial step in the assessment and evaluation of the toxic characteristic of compounds. To establish the LD_{50}, the Karber arithmetic method was used that is based on the formula:[18]

$$LD_{50} = LD_{100} - \frac{\Sigma(a \times b)}{n} \quad (1)$$

where a is the difference between two successive doses of the tested compound; b is the arithmetic average of the animals from two successive series that died; n is the number of animals per group and LD_{100} is the 100 % lethal dose. The values of LD_{50} for TSP-81, the xanthine intermediary D6, theophylline and acetaminophen are given in Table III.

TABLE III. LD_{50} values for TSP-81, its intermediary (D6) and the parent compounds (theophylline and acetaminophen)

Compound	LD_{50} / mg kg^{-1}			
	24 h	48 h	72 h	Average
TSP-81	456	456	420	444
D6	439	439	390	423
Theophylline	205	205	190	200
Acetaminophen	351	351	312	338

The LD_{50} of TSP-81 that combines theophylline with acetaminophen using nitrate ester alkyl chain as a spacer was 444 mg kg^{-1}, which means that it was slightly less toxic than its intermediary, D6 (LD_{50} 423 mg kg^{-1}) but it is much less toxic than its parent compounds. TSP-81 is 2.2 times less toxic than theophylline and 1.3 less toxic than acetaminophen. These results are important because it is known that both theophylline and acetaminophen are associated with several side effects, especially when they are used in chronic treatment.

Cotton pellet-induced granulation inflammation

The cotton pellet-induced granuloma is widely used to assess the transudative and proliferative components of chronic inflammation.[24] The implanted material induces a host inflammatory response and modulates the release of inflammatory mediators that finally leads to tissue proliferation and granular formation.[25] The weight of the wet cotton pellets is correlated with transude material and the weight of the dry pellets is correlated with the granuloma tissue formation. A decreasing of granuloma tissue formation is an indicator of the antiproliferative effect of an anti-inflammatory drug. The effect of the tested compounds (TSP-81, D6, theophylline and acetaminophen) in reference with the control group (that received only 0.5 % carboxymethylcellulose), on the cotton pellet-induced granuloma in rats are given in Table IV. After six days, the dry weight of the granuloma tissue surrounding the cotton pellets was significantly lower ($P < 0.05$) for the group treated with TSP-81, compared to the control group, but also compared with its intermediary (D6) and parent compounds. TSP-81 exhibited a value of 15.32±1.09 related to 63.46±1.16 (control), 31.68±1.23

TABLE IV. The effects of the tested compounds on granuloma tissue formation (data represent average ± *SEM*, $n = 6$. *$P < 0.05$ *vs.* control)

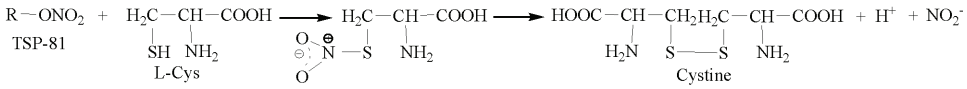
Compound	Dose mg kg^{-1}	Weight of the dry cotton pellet, mg	Weight of the granuloma tissue, mg	Inhibition level %
TSP-81	90	76.82±1.14*	15.32±1.09*	75.85
D6	85	91.38±1.18*	31.68±1.23*	50.07
Theophylline	40	100.25±1.12*	38.77±1.20*	38.90
Acetaminophen	70	121.36±1.10*	60.28±1.22*	5.01
Control	Vehicle	124.46±1.15	63.46±1.16	–

(D6), 38.77±1.20 (theophylline) and 60.28±1.22 (acetaminophen). These results support the conclusion that TSP-81 is approximately 2 times more active than theophylline and 4 times more active than acetaminophen as an anti-inflammatory drug.

Detection of nitrite

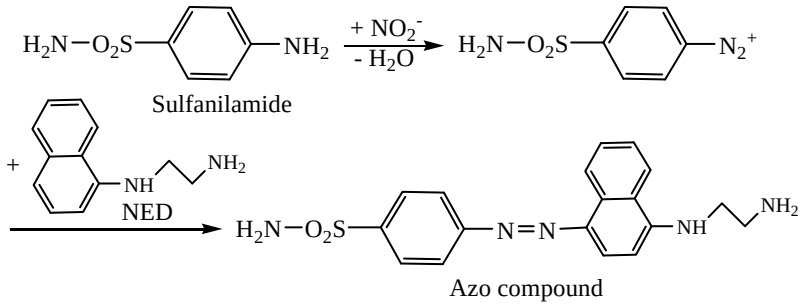
The nitric oxide releasing property of TSP-81 was assessed in phosphate buffer, pH 7.4, in the presence of L-cysteine, as a source of SH groups, and it was calculated based on the nitric oxide released from standard sodium nitrite solution.

It is known that nitric oxide donors release NO through enzymatic and non-enzymatic pathways. The non-enzymatic pathway involves chemical reaction with acid, alkali, metals and compounds with SH groups.[26] The nucleophilic attack of L-cysteine on the nitrate group of TSP-81 resulted in the formation of a thionitrate as an intermediate to the formation of cysteine and nitrite during the next step (Scheme 2).



Scheme 2. The mechanism of the NO release from TSP-81.

The released nitrite was detected using the Griess reagent system that uses sulphanilamide and *N*-(1-napthyl)ethylenediamine dihydrochloride (NED) under acidic conditions. The red–pink azo compound was detected spectrophotometrically (Scheme 3).



Scheme 3. Chemical reactions involved in the measurement of NO_2^- using the Griess reagent system.

The absorbance of the TSP-81 solution increased with the concentration and the amount of NO released related to a theoretical maximum release of 1 mol NO mol^{-1} of test compound was found to be around 0.5 mol (Table V). The results

confirm the ability of the TSP-81 to release NO and also the implication of the NO in the expression of the anti-inflammatory effect of the tested compound.

TABLE V. The nitric oxide releasing effect of the TSP-81 (data represent average ± *SEM*, $n = 3$, $P < 0.05$)

Volume, mL	Concentration, μg mL^{-1}	Absorbance	NO_2^- released, mol
0.05	330	0.2748±0.0052	0.4763±0.04314
0.1	650	0.57313±0.0144	
0.2	1250	0.95207±0.0310	

CONCLUSIONS

A new xanthine nitric oxide donor (TSP-81) was synthesized and characterized from spectral, thermal and biological viewpoints. The thermo-oxidative decomposition of the TSP-81 occurred in four steps. As was expected, this new NO-donor compound was less stable than its intermediate (D6) and parent drugs (theophylline and acetaminophen). The results of the biological evaluation of the compound support its lower toxicity, its higher anti-inflammatory effect and its ability to release nitric oxide. As a result, it could be supposed that the anti-inflammatory effect of TSP-81 is due to the inhibition of phosphodiesterase activity and also to its nitric oxide releasing properties. In conclusion, TSP-81 could become an important drug in the management of asthma and chronic obstructive pulmonary diseases.

SUPPLEMENTARY MATERIAL

Physical, analytic and spectral data for the synthesised compounds are available electronically from http://www.shd.org.rs/JSCS/, or from the corresponding author on request.

ИЗВОД

СИНТЕЗА, КАРАКТЕРИЗАЦИЈА И ИСПИТИВАЊЕ БИОЛОШКЕ АКТИВНОСТИ НОВОГ ДОНОРА АЗОТ-МОНОКСИДА

LENUTA PROFIRE[1], MARIA APOTROSOAEI[1], ANCA OPREA[2], MIHAI BREBU[2], FLORENTINA LUPASCU[1], CATALINA ELENA LUPUSORU[3] и CORNELIA VASILE[2]

[1]Department of Pharmaceutical Chemistry, Faculty of Pharmacy, "Grigore T. Popa" University of Medicine and Pharmacy, 16 University Str., 700115, Iasi, Romania, [2]Department of Physical Chemistry of Polymers, "P. Poni" Institute of Macromolecular Chemistry, Romanian Academy, 41A Grigore Ghica Voda Alley, 700487, Iasi, Romania и [3]Department of Pharmacology, Faculty of Medicine, "Grigore T. Popa" University of Medicine and Pharmacy, 16 University Str., 700115, Iasi, Romania

Описана је синтеза ксантинског донора (TSP-81) азот-моноксида. Једињење је осмишљено да садржи две структурне целине – теофилин (1,3-диметилксантин) и ацетаминофен (4-хидроксиацетанилид), повезане линкером који садржи извор азот-моноксида. Једињење је окарактерисано микроанализом, спектралним методама ^{1}H-NMR, ^{13}C- NMR, FT-IR, UV–Vis као и TG и DTG техникама. Термални профил показује да се TSP-81 топи у четири корака, уз распадање, од којих су најважнији други корак (детектован губитак масе 17,6 %) и трећи корак (регистрован губитак масе 30,4 %). Такође, испитана је токсичност, анти-инфламаторни ефекат и способност отпуштања

азот-моноксида. Биолошки тестови показују да TSP-81 има ниску токсичност и изражен анти-инфламаторни ефекат, у поређењу са теофилином и ацетаминофеном, једињењима из којих је изведен. TSP-81 је око 2 пута активнији од теофилина и око 4 пута активнији од ацетаминофена. Осим тога, резултати указују да ослобађање азот-моноксида доприноси анти-инфламаторном ефекту.

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