processes

MDPI

Article

Assessment of a Diverse Array of Nitrite Scavengers in Solution and Solid State: A Study of Inhibitory Effect on the Formation of Alkyl-Aryl and Dialkyl *N*-Nitrosamine Derivatives

Miha Homšak [1,†], Marko Trampuž [1,†], Klemen Naveršnik [1], Zoran Kitanovski [1], Mateja Žnidarič [1], Markus Kiefer [2] and Zdenko Časar [1,3,*]

[1] Sandoz Development Center Slovenia, Lek Pharmaceuticals d.d., Kolodvorska cesta 27, 1234 Mengeš, Slovenia
[2] Global Manufacturing Science & Technology, Novartis Technical Operations, Fabrikstrasse 2, Novartis AG, 4056 Basel, Switzerland
[3] Faculty of Pharmacy, University of Ljubljana, Aškerčeva cesta 7, 1000 Ljubljana, Slovenia
* Correspondence: zdenko.casar@sandoz.com or zdenko.casar@ffa.uni-lj.si; Tel.: +386-1-5802079
† These authors contributed equally to this work.

Abstract: The ubiquitous presence of mutagenic and potentially carcinogenic *N*-nitrosamine impurities in medicines has become a major issue in the pharmaceutical industry in recent years. Rigorous mitigation strategies to limit their amount in drug products are, therefore, needed. The removal of nitrite, which is a prerequisite reagent for the *N*-nitrosation of amines, has been acknowledged as one of the most promising strategies. We have conducted an extensive literature search to identify nineteen structurally diverse nitrite scavengers and screened their activity experimentally under pharmaceutically relevant conditions. In the screening phase, we have identified six compounds that proved to have the best nitrite scavenging properties: ascorbic acid (vitamin C), sodium ascorbate, maltol, propyl gallate, *para*-aminobenzoic acid (PABA), and L-cysteine. These were selected for investigation as inhibitors of the formation of *N*-methyl-*N*-nitrosoaniline (NMA) from *N*-methylaniline and *N*-nitroso-*N*′-phenylpiperazine (NPP) from *N*-phenylpiperazine in both solution and model tablets. Much faster kinetics of NMA formation compared to NPP was observed, but the former was less stable at high temperatures. Vitamin C, PABA, and L-cysteine were recognized as the most effective inhibitors under most studied conditions. The nitrite scavenging activity does not directly translate into *N*-nitrosation inhibitory effectiveness, indicating other reaction pathways may take place. The study presents an important contribution to identifying physiologically acceptable chemicals that could be added to drugs to prevent *N*-nitrosation during manufacture and storage.

Keywords: nitrite; *N*-nitrosamine; impurity; scavenger; vitamin C; amino acids; stability; final dosage forms

Citation: Homšak, M.; Trampuž, M.; Naveršnik, K.; Kitanovski, Z.; Žnidarič, M.; Kiefer, M.; Časar, Z. Assessment of a Diverse Array of Nitrite Scavengers in Solution and Solid State: A Study of Inhibitory Effect on the Formation of Alkyl-Aryl and Dialkyl *N*-Nitrosamine Derivatives. *Processes* **2022**, *10*, 2428. https://doi.org/10.3390/pr10112428

Academic Editor: Alina Pyka-Pająk

Received: 27 October 2022
Accepted: 12 November 2022
Published: 17 November 2022

1. Introduction

In 2018, *N*-nitrosodimethylamine (NDMA, Figure 1a) was found as a contaminant in angiotensin-II-receptor antagonist (sartans) drugs [1–6]. This triggered industry-wide investigations into the origin of this contamination, which revealed that many active pharmaceutical ingredients (APIs) and drug products are contaminated with low molecular weight *N*-nitrosamines such as NDMA, *N*-nitrosodiethylamine (NDEA), diisopropyl-*N*-nitrosamine (DIPNA), ethylisopropyl-*N*-nitrosamine (EIPNA), 4-(methyl)[(nitroso)amino]butanoic acid (NMBA) (Figure 1a), and others [7]. A variety of simple *N*-nitrosamines may be formed from APIs, such as NDMA, which has been found in hydrochlorothiazide, ranitidine, and metformin [8]. Moreover, soon after it was realized that APIs that contain at least one secondary amine moiety are also prone to *N*-nitrosation, which extended *N*-nitrosamine challenges to a new dimension, because many APIs contain this structural motif [9]. Indeed, more structurally complex *N*-nitrosamines, formed by the direct *N*-nitrosation of APIs, are

posing a serious concern (as exemplified by the API varenicline, Figure 1b) and are collectively called nitrosamine drug substance-related impurities (NDSRIs) [9,10]. Therefore, novel guidelines had to be adopted from the regulatory agencies regarding strict control over the amounts of *N*-nitrosamines in drugs [7].

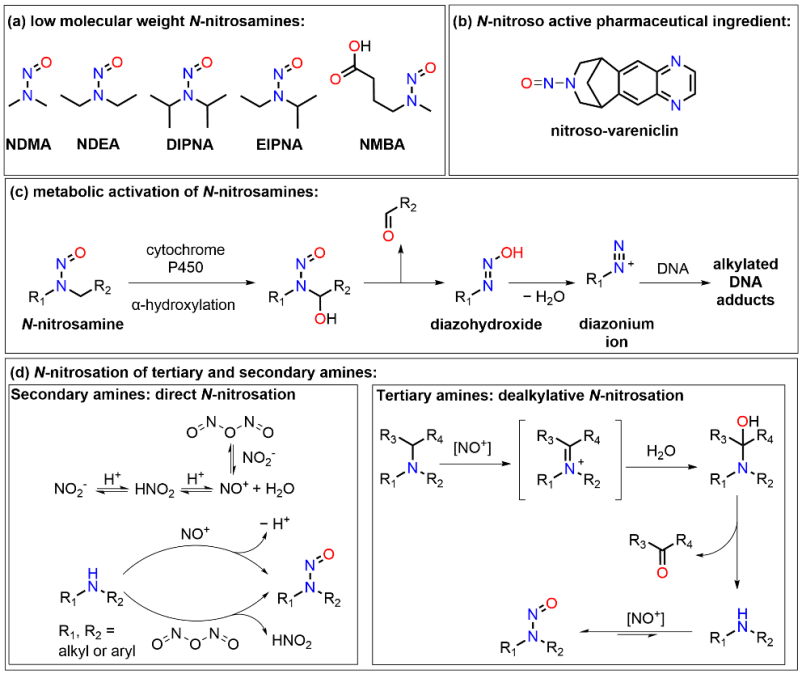

Figure 1. (**a**) Most common low molecular weight *N*-nitrosamines; (**b**) *N*-Nitroso-varenicline; (**c**) Metabolic transformation of *N*-nitrosamines to carcinogenic diazonium ions; (**d**) Mechanism of *N*-nitrosation of secondary and tertiary amines.

N-Nitrosamines are a heterogeneous group of probable human carcinogenic chemical compounds which contain a nitroso group directly bound to a nitrogen atom. Upon metabolic activation by cytochrome P450-mediated hydroxylation on the *alpha*-carbon and subsequent aldehyde elimination, they form highly reactive diazonium species, which may alkylate deoxyribonucleic acid (DNA) and lead to mutations and oncogenesis (Figure 1c) [11–16]. *N*-Nitrosamines have been proven carcinogenic in laboratory animals and, depending on their structure, many of them are classified in the International Agency for Research on Cancer (IARC) monograph as either carcinogenic (Group 1; *N*′-nitrosonornicotine (NNN), 4-(*N*-nitrosomethylamino)-1-(3-pyridyl)-1-butanone (NNK)), probably carcinogenic (Group 2A; NDEA, NDMA), and possibly carcinogenic (Group 2B; *N*-nitrosopiperidine, *N*-nitrosomethylethylamine, *N*-nitrosodiethanolamine (NDELA), *N*-nitrososarcosine, *N*-nitrosomethylvinylamine, *N*-nitrosomorpholine (NMOR), 3-(*N*-nitrosomethylamino) propionitrile, *N*-nitrosodi-*n*-propylamine, *N*-nitrosodi-*n*-butylamine, *N*-nitrosopyrrolidine) to humans as well [8,12,17,18]. They are ubiquitous in the environment, as they may be found in water [19,20], food [12], air [21,22], soil [23–25], cigarette smoke [26], rubber [27], cosmetics [28], and medicines [20,29,30]. As highlighted above, a number of medicinal products have been found to be contaminated with unacceptably high levels of *N*-nitrosamines, which has led to the recalls of several products in recent years [2–6,8].

The mechanisms of *N*-nitrosamine formation have been the subject of investigation for over 40 years, especially in regard to nitrite and nitrate found in food. Their presence in medicinal products has, moreover, led to renewed interest in understanding the risk of their formation in vivo, in medicines, and even more so in their structure-activity relationships regarding the molecular mechanism of their chemical toxicology. Apart from the general CYP-450 activation pathway, only little is known about whether aromatic and aliphatic secondary amines or bulky structures exhibit similar DNA alkylating potency. However,

this information is important for establishing limits using the so-called read-across comparisons, where data gaps on the substance of interest are filled using data on closely related substances. The key precursors for the generation of *N*-nitrosamines are tertiary and, especially, secondary amines in the presence of nitrite (NO_2^-; Figure 1d). However, tertiary amines represent a lower risk for *N*-nitrosamine formation, due to approximately 1000-times lower reactivity compared to secondary amines [20]. Under acidic conditions, nitrite is transformed into notoriously short-lived nitrous acid (HNO_2), which further reacts with amines to form *N*-nitroso products via either the highly electrophilic nitrosonium cation (NO^+) or dinitrogen trioxide (N_2O_3) [20,31] (Figure 1d). Alternatively, secondary amines can be directly *N*-nitrosated by nitric oxide (NO) in the presence of oxygen [32]. *N*-Nitrosamines may be formed in medicinal products both during manufacturing and storage. Nitrites can be present in a range of pharmaceutical excipients [33,34] and water as impurities, but are also sometimes intentionally used during the chemical synthesis of APIs. Secondary and tertiary amines are ubiquitous, either as structural fragments in APIs or chemical reagents (solvents such as dimethylamine, diethylamine, or *N*-methyl-2-pyrrolidone) and precursors in API synthesis which increases the risk for *N*-nitrosamine formation in drug manufacturing.

Based on the understanding of *N*-nitrosamine formation, pharmaceutical companies are nowadays stimulated to adopt mitigation strategies to inhibit their formation [10]. Due to the crucial role of nitrites, the strict control of the presence of nitrite impurities in water, excipients, and during chemical synthesis, for example, by means of a supplier qualification program, presents the most obvious option [10]. This is, however, laborious and often difficult to ensure practically [34]. A second approach is the use of basic excipients (for example, Na_2CO_3) that modify the microenvironment in medicinal products to make it less acidic and, thus, decrease the rate of any potential *N*-nitrosation reaction, but care must be taken to assure that neither the stability nor dissolution or uptake of API are affected. A third possible strategy would be the use of excipients that prevent *N*-nitrosation by scavenging nitrite anion prior to its reaction with amines. Such compounds could exert their scavenging action already within medicinal products during the manufacturing process and storage, not only in vivo in the stomach after the ingestion of such medicines [10].

The addition of nitrite scavengers to various products for *N*-nitrosation inhibition has been widely studied in the past and several compounds with such an action have already been identified, with ascorbic acid (vitamin C) being the most well-known. A number of studies have proven its activity to inhibit the *N*-nitrosation reaction via nitrite reduction in solution [35,36], tobacco matrix systems [37], cosmetics [38], and drugs [39]. However, its activity may be worsened considerably by lipids [40,41]. Related substances, such as erythorbic acid, sodium ascorbate, and ascorbyl palmitate, have also been shown to be effective [42,43]. Furthermore, it has been demonstrated that a wide variety of other different antioxidants were able to inhibit *N*-nitrosamine formation, including alpha-tocopherol [39] and various other phenols and phenolic acids [44–46], which presumably work by an oxidation/nitrosation mechanism. Other compounds that have successfully decreased the rate of the *N*-nitrosation reaction through nitrite scavenging include, but are not limited to, urea [35], sulfamic acid [47,48], hydrazoic acid [48,49], various amines [48,50–53], and amino acids [37,39,54]. To the best of our knowledge, the only study so far that has evaluated the addition of various nitrite scavengers in medicinal products has been the recent proof of concept study by Nanda et al. [39]. Five different antioxidants as additives in tablets and three different amino acids in solution were evaluated for their ability to inhibit the *N*-nitrosation of model API 4-phenylpiperidine hydrochloride. All of them led to a decrease in the amount of the formed *N*-nitrosated product, proving that nitrite scavenging may indeed be a promising strategy for *N*-nitrosamine risk mitigation.

The goal of the presented study was to build upon the mentioned proof of concept study. We first conducted an extensive literature search of potential compounds with nitrite scavenging abilities that are acceptable for use in medicinal products (listed in the FDA's Inactive Ingredients Database), are commonly present in food (resveratrol) or are

known human metabolites (PABA) [55,56]. The selected 19 compounds were then thoroughly screened experimentally for their ability to deplete the nitrite in aqueous solutions under various reaction conditions, most critical for the *N*-nitrosation reaction. The best performing compounds were then evaluated for their inhibitory effect on *N*-nitrosamine formation both in solution and in solid state on two model APIs, *N*-methylaniline (MA) and *N*-phenylpiperazine (PP), as representative alkyl-aryl and dialkyl secondary amines, which are common and the most vulnerable structural motifs in APIs. Examples of APIs containing an MA motif are furosemide and retigabine, whereas a PP motif can be found in paroxetine and vortioxetine.

2. Materials and Methods

2.1. Materials

All chemicals and solvents were purchased from Sigma-Aldrich (St. Louis, MO, USA) or VWR (Randor, PA, USA) unless otherwise stated and used without further purification. The Milli-Q (MQ) water for experiments and buffer preparation was obtained from the laboratory purification system Millipore Milli-Q lab water system (MilliporeSigma, Burlington, MA, USA). NO_2^- concentration in the solution was measured with 2–80 mg/L nitrite colorimetric test strips MQuant® (MilliporeSigma, Burlington, MA, USA). Microcrystalline cellulose (MCC; Vivapur 101 grade) was sourced from JRS Pharma (Rosenberg, Germany), crospovidone (PVP; Polyplasdone grade) was sourced from Ashland (Wilmington, DE, USA), and magnesium stearate (MS; Ligamed MF-2-V grade) was obtained from Peter Greven (Bad Münstereifel, Germany).

2.2. Scavenger Screening Experiments

Two 50 mM phosphate buffers were prepared. The pH 3.0 buffer was made by mixing 85% H_3PO_4 with MQ water and adjusting to the desired pH with NaOH, whereas the pH 5.0 buffer was prepared by dissolving $NaH_2PO_4 \times H_2O$ in MQ water and adjusting to the desired pH with NaOH. Stock solutions of all the chosen scavengers (43.48 mM, 20 Eq.) were prepared by dissolving the appropriate amounts of each of the 19 compounds in either the pH 3.0 or pH 5.0 phosphate buffer; these were stored at 2–8 °C until further use. Similarly, $NaNO_2$ was dissolved in each of the buffers to prepare 4.348 mM (200 ppm, 2 Eq.) stock solutions; the nitrite solution was always prepared fresh, immediately before use. To perform the experiments, equal volumes of a scavenger solution and the nitrite solution (both either pH 3.0 or pH 5.0, molar ratio NO_2^-: scavenger = 1:10; final concentrations 2.174 and 21.74 mM, respectively) were mixed and incubated for the desired amount of time at 20 °C or 50 °C with constant agitation set at 750 rpm. The disappearance of the NO_2^- ion over time was measured at set timepoints with nitrite colorimetric test strips.

2.3. Model N-Nitrosamine Synthesis

Model *N*-nitrosamines *N*-nitroso-*N*-methylaniline (NMA), and *N*-nitroso-*N*′-phenylpiperazine (NPP) were prepared from MA and PP according to the published literature procedure [57].

Hydrochloride salts of MA (MA × HCl) and PP (PP × HCl) for the solid-state experiments were prepared from MA and PP according to the previously published procedure as well [58].

2.4. Scavenger Evaluation on Inhibition of Model API N-Nitrosation in Solution

Twelve different reactions were performed to assess the impact of five different scavengers (plus a control without any scavenger) on two different model APIs. A 4 mL vial with a magnetic stirring bar was charged with 500 µL of 8.0 mM solution of model API (MA or PP) in 10 mM phosphate buffer pH 3.0 and 1000 µL of 88.0 mM solution of nitrite scavenger in 10 mM phosphate buffer pH 3.0. Afterwards, 500 µL of 8.8 mM solution of sodium nitrite in 10 mM phosphate buffer pH 3.0 was added to achieve the final concentrations of model API: nitrite: scavenger 2.0, 2.2, and 44.0 mM (molar ratio 1:1.1:22). For control reactions, only a buffer was added instead of a scavenger solution. Reaction mixtures were stirred at 200 rpm at 25 °C. Mixtures with MA were sampled for quantification of

the formed NMA immediately after mixing, after 30 min, 1, 2 and 8 h. Mixtures with PP were sampled for quantification of the formed NPP immediately after mixing, after 2, 4, 8 and 24 h. An amount of 50 µL of the sample was added to 950 µL of 0.53 mM NaOH solution and analyzed by LC-MS. The disappearance of the NO_2^- ion over time was measured at the same timepoints with nitrite colorimetric test strips. All the reactions were performed in duplicates.

2.5. Scavenger Evaluation on Inhibition of Model API N-Nitrosation in Tablets

Twenty-eight different powder blends were prepared as combinations of two different model APIs, six different scavengers (plus a control without any scavenger), and two different quantities of nitrite in excipients. Two tablets were compressed for each blend.

The tablets were prepared according to the mass percentages defined in Table 1. First, nitrite-spiked MCC was prepared by adding 4 µg/mL sodium nitrite aqueous solution to MCC to achieve 2 ppm concentration of nitrite. The same amount of water was added to non-spiked MCC as the control. Both mixtures were mixed using a mortar and pestle until a visually homogenous wet powder. PVP and MS were added to each mixture and mixed using a mortar and pestle. Both mixtures were divided into two equal parts and MA × HCl and PP × HCl were added in powder form, respectively. Scavenger solution (1 mL, 12 mg/mL) in water/ethanol (50/50) was then added to portions of each mixture (1 mL of water/ethanol was added to the control experiment with no scavenger) and mixed with a mortar and pestle. The powders were dried under nitrogen at 50 °C for 8 h and compressed into 0.2 g tablets using a 15-tonne manual hydraulic press, with a 13 mm diameter die (Specac Ltd., Orpington, UK). The tablets were halved and subjected to stress testing at 50 °C and 75% relative humidity (RH) open dish for 28 days in a chamber KBF 115 (Binder Gmbh, Tuttlingen, Germany). One tablet per blend was analyzed before stability testing and two tablet halves after the testing. For NPP quantification analysis, the tablets were weighed and suspended in 50 mM ammonium bicarbonate buffer pH 7.0 to a 5.0 mg/mL target concentration of PP. The buffer was prepared by dissolving the appropriate amount of ammonium bicarbonate in demineralized water and the pH was set using formic acid. The same procedure was used for NMA quantification where 20% of acetonitrile was added to the ammonium bicarbonate buffer to obtain sufficient solubility of MA. The suspensions were shaken manually until the tablets visibly completely disintegrated and then centrifuged at 4000 rpm for 5 min. Finally, 1 mL of clear supernatant was transferred into an HPLC vial and analyzed by LC-MS.

Table 1. Composition of tablets.

Ingredient	Control Tablets [w/w%]	NO_2^- Spiked Control Tablets [w/w%]	Tablets with Scavenger [w/w%]	NO_2^- Spiked Tablets with Scavenger [1] [w/w%]
API (MA × HCl/PP × HCl)	8.3	8.3	8.2	8.2
Scavenger	0	0	0.82	0.82
MS	0.83	0.83	0.82	0.82
MCC	83	0	82	0
NO_2^- spiked MCC	0	83	0	82
PVP	8.3	8.3	8.2	8.2

[1] The molar ratio of API: added nitrite: scavenger was 24.021:1:1626–2847 for MA × HCl and 17.362:1:1626–2847 for PP × HCl. The excess of scavenger depended on its molar mass and was highest for **16** (molar mass 121.15 g/mol) and lowest for **7** (molar mass 212.20 g/mol). The amount of nitrite was likely higher due to its presence in excipients and the excess of API and scavenger was consequently lower.

2.6. Liquid Chromatography-Mass Spectrometry Method for NMA/NPP Quantification

The quantification of NMA and NPP in the solution and tablet samples was done using a Waters Acquity ultra-high performance liquid chromatograph (UHPLC) coupled to a Waters Xevo triple quadrupole mass spectrometer (Waters, Milford, MA, USA). The separation of MA, PP, and their nitroso derivates was achieved on an Acquity UPLC BEH

C18, 50 × 2.1 mm i.d., 1.7 µm column (Waters) by using a mobile phase consisting of 0.1% (*v*/*v*) aqueous formic acid (mobile phase A) and methanol (mobile phase B, MP.B), at a flow rate of 300 µL/min. Gradient elution was employed as follows: 0–1 min: 5% MP.B; 10 min: 40% MP.B; 10.5 min: 70% MP.B; 12 min: 70% MP.B; 12.5 min: 5% MP.B; and 16 min: 5% MP.B (column re-equilibration). The injection volume and the column temperature were set to 10 µL and 25 °C, respectively. The detection and quantification of the analytes was achieved by using positive polarity ESI-MS/MS in the selected reaction monitoring (SRM) mode. An optimization of the ESI source parameters was done to ensure most sensitive analyte signals and stable electrospray. The final optimized ESI parameters were as follows: +2 kV for ESI capillary voltage; 20 V for the cone voltage; 0 and 650 L/h for the cone and desolvation gas flow, respectively; 0.15 mL/min for the collision gas flow; and 350 °C for the desolvation temperature. High-purity nitrogen was used as a cone and desolvation gas, while high-purity argon was employed as a collision gas. SRM transitions *m*/*z* 137 → *m*/*z* 66 and *m*/*z* 192 → *m*/*z* 120 were used for the selective detection of NMA and NPP, respectively. The dwell time of the SRM transitions was set to 300 ms. MassLynx 4.2 software (Waters) was used for the acquisition and analysis of the LC/MS data. For the representative chromatograms, see Figures S7 and S8.

3. Results

3.1. Scavenger Screening Experiments

Our work started with a thorough literature review of the available and known nitrite scavengers. We were able to produce a list of 19 compounds that have been shown to react well with nitrite and are toxicologically acceptable. Most are also included on the FDA Inactive Ingredients Database. Their scavenging ability was first assessed in a series of screening experiments at two different temperatures and pHs in order to identify the compounds that show the best scavenging reactivity with nitrite. The scavengers used in the experiments are listed in Table 2.

Table 2. The scavengers included in the initial screening experiments.

Scavenger Number	Scavenger Name	Scavenger Number	Scavenger Name
1	Ascorbic acid [37,39,59]	**11**	Arginine [60]
2	Sodium ascorbate [39]	**12**	Lysine [39,60]
3	Ascorbyl palmitate [42,44,61]	**13**	Histidine [39]
4	α-tocopherol [37]	**14**	Urea [48,62]
5	Maltol [44]	**15**	PABA [52]
6	Resveratrol [63,64]	**16**	L-Cysteine [37]
7	Propyl gallate [42,61]	**17**	Sodium sulfite [61,62]
8	BHA [65]	**18**	Ammonium sulfate [53,62]
9	BHT [65]	**19**	Ammonium chloride [53]
10	Glycine [39,60]	**Ctrl**	NO_2^- [1]

[1] Nitrite without any added scavenger was used as the control in each of the screening experiments.

Large differences in reactivity were observed between the different scavengers (Figure 2). Scavengers **1**, **2** and **16** produced the most striking results—no leftover nitrite could be detected in 1–2 h in the reactions performed at pH 3.0 and 20 °C. Scavenger **5** took 8 h to completely remove the nitrite from the solution, whereas the other tested compounds needed at least 24 h (**6**, **7**, **15**) or failed to completely scavenge the nitrite. Importantly, the nitrite concentration steadily decreased over time even in the control that did not contain any scavengers due to its transformation to nitrous acid and subsequent decomposition to NO and NO_2 [1,66]. Similar results were obtained with the reactions run at pH 5.0 and 20 °C, with the reaction kinetics unsurprisingly being slower due to the higher pH (Figure S1 in Supplementary material). The same observation was made when the same experiment was run at the two pHs at 50 °C—the reactions were approximately 10–times faster than at 20 °C when comparing the respective pH conditions (Figures S2 and S3 in Supplementary material).

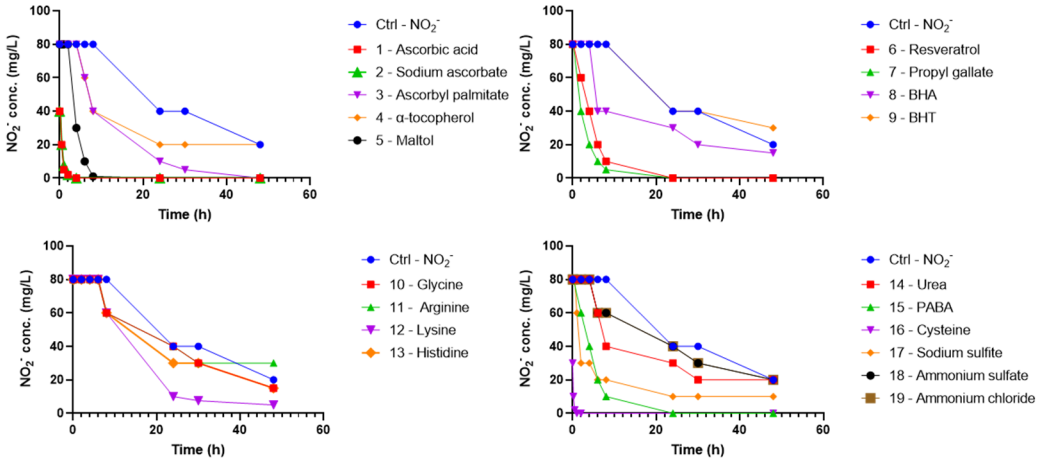

Figure 2. The result of the screening experiment with the 19 shortlisted scavengers, showing the disappearance of NO_2^- over time in the presence of various scavengers. Nitrite: scavenger 1:10 (molar ratio), all reactions carried out at 20 °C in pH 3.0 phosphate buffer.

The results obtained with scavengers **3**, **4**, **6**, **7**, **8**, **9** and **15** are not entirely representative due to their (very) poor aqueous solubility. To circumvent this problem, we attempted to run a similar screening experiment in the co-solvent mixtures of the two buffers with DMSO/EtOH/*i*-PrOH, but the high fraction of organic solvent (75% *v*/*v*) required to solubilize the water-insoluble compounds precluded the scavenging reactions from running (Figure S4 in Supplementary material).

Despite the solubility issue, the screening experiments allowed us to identify a smaller subset of six scavengers to be evaluated in the solid-state experiments with model APIs. Therefore, scavengers **1**, **2**, **5**, **7**, **15** and **16** (Figure 3) were utilized due to their high reactivity. For the solution experiments, **2** was omitted (as it forms the same chemical species at pH 3.0 as **1**) for a total of five scavengers utilized in those investigations.

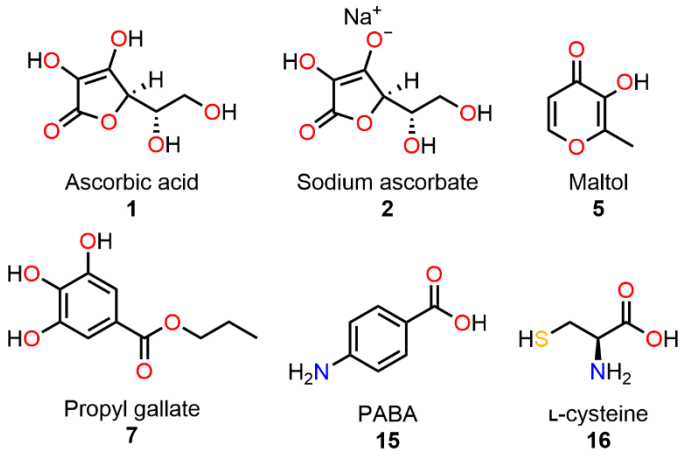

Figure 3. Chemical structures of the six selected scavengers **1**, **2**, **5**, **7**, **15** and **16** for evaluation of their *N*-nitrosation inhibition activity in solid-state experiments. Scavenger **2** was omitted for evaluation in solution experiments.

3.2. Scavenger Evaluation on Inhibition of Model API N-Nitrosation in Solution

Subsequently, we have studied the impact of the selected scavengers on the conversion of MA to NMA and PP to NMP under acidic conditions (Figure 4).

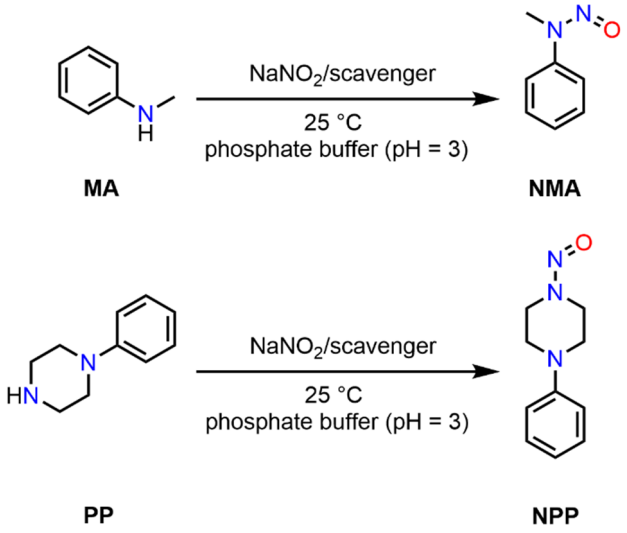

Figure 4. Reaction between MA or PP with nitrite at pH = 3.

The impact of the five selected nitrite scavengers on the *N*-nitrosation of MA and PP in the solution is shown in Figures 5 and 6. When no scavenger was present, MA was quantitatively *N*-nitrosated to NMA in the solution within 30 min at 25 °C (dark blue curve in Figure 5), while only around 5% of PP was transformed to NPP even after 24 h (blue curve in Figure 6). Some NMA could already be detected in the reaction mixtures immediately after the reactions were initiated, indicating very fast reaction kinetics.

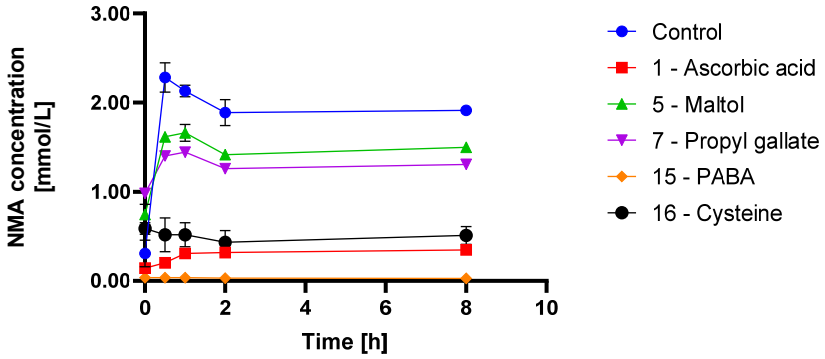

Figure 5. The results of *N*-nitrosation inhibition of MA in solution with the five selected scavengers **1**, **5**, **7**, **15** and **16**. Initial MA concentration was 2 mM, the molar ratio of MA: nitrite: scavenger was 1:1.1:22, and all reactions were carried out at 25 °C in pH 3.0 phosphate buffer.

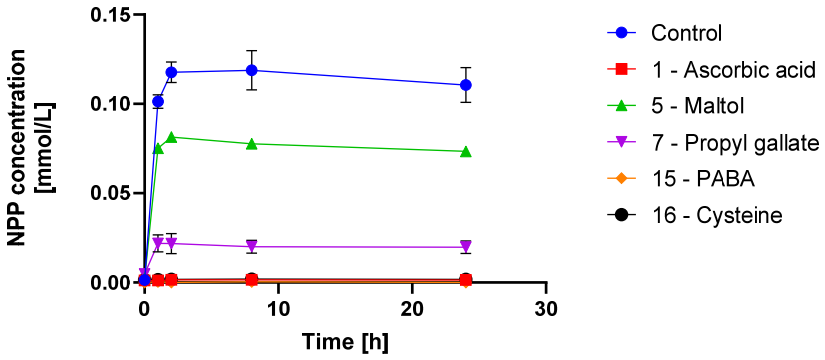

Figure 6. The results of *N*-nitrosation inhibition of PP in solution with the five selected scavengers **1**, **5**, **7**, **15** and **16**. Initial PP concentration was 2 mM, the molar ratio of PP: nitrite: scavenger was 1:1.1:22, and all reactions were carried out at 25 °C in pH 3.0 phosphate buffer.

Compared to the control reaction mixtures, all five scavengers decreased the amount of NMA and NPP to some extent. *Para*-aminobenzoic acid (**15**) was the most effective scavenger as only traces of NMA and no NPP could be detected in the reaction mixtures (orange curves). Ascorbic acid (**1**) and L-cysteine (**16**) were also highly effective in the prevention of NPP formation, as they decreased its concentration by more than 98% compared to the control reaction (red and black curves, respectively). Their activity against MA *N*-nitrosation was also significant, albeit lower, as shown in Figure 5 (75–90% less compared to control reaction). Propyl gallate (**7**) was more effective in decreasing the formation of NPP than NMA (purple curves). Maltol (**5**) was the least effective scavenger as it decreased the amount of NMA and NPP only by approximately 20–35% (green curves).

3.3. Scavenger Evaluation on Inhibition of Model API N-Nitrosation in Tablets

Tables 3 and 4 present the impact of the selected six scavengers on the prevention of the *N*-nitrosation of MA × HCl and PP × HCl in the tablets during the 28-day stress testing at 50 °C and 75% RH. Two completely different trends may be observed by comparing both model APIs. *N*-Nitrosation of MA × HCl had already occurred to a considerable extent during granulation and tableting, as NMA was already detected in all the tablets prior to stress testing. Furthermore, no NMA may be detected in any of the samples after stress testing (including the control tablets without any scavenger), indicating that NMA is not stable during stress testing and was either degraded or lost to evaporation. The opposite holds for the transformation of PP × HCl to NPP, where some tablets with PP × HCl contained NPP prior to stress testing, and all of them contained it after stress testing.

Table 3. The results of MA × HCl *N*-nitrosation inhibition in the tablets (28-day stress testing at 50 °C and 75% RH) with the six selected scavengers **1**, **2**, **5**, **7**, **15** and **16**, reported in ppm per initial mass of MA. The % value in brackets represents inhibition efficiency [1], which was calculated for each column and scavenger individually.

Scavenger Number	Initial NMA [ppm] no NO_2^- Spiking	Final NMA [ppm] no NO_2^- Spiking [3]	Initial NMA [ppm] with NO_2^- Spiking	Final NMA [ppm] with NO_2^- Spiking [3]
Ctrl [2]	16.82	n.d. [4]	33.78	n.d. [4]
1	14.84 (−11.8%)	n.d. [4]	46.75 (+38.4%)	n.d. [4]
2	14.47 (−13.9%)	n.d. [4]	28.66 (−15.2%)	n.d. [4]
5	20.74 (+23.4%)	n.d. [4]	54.12 (+60.2%)	n.d. [4]
7	21.23 (+26.3%)	n.d. [4]	97.31 (+188.1%)	n.d. [4]
15	21.69 (29.0%)	n.d. [4]	28.60 (−15.3%)	n.d. [4]
16	25.95 (+54.3%)	n.d. [4]	36.76 (+8.8%)	n.d. [4]

[1] Inhibition efficiency = 100% × (NMA amount with scavenger − NMA amount in control)/(NMA amount in control). [2] No added scavenger was used as the control for each of the experiments. [3] Values are reported as averages of measurements in two tablets. [4] Values below LOQ of 40 ppb.

Table 4. The results of PP × HCl *N*-nitrosation inhibition in the tablets (28-day stress testing at 50 °C and 75% RH) with the six selected scavengers **1**, **2**, **5**, **7**, **15** and **16**, reported in ppm per initial mass of PP. The % value in brackets represents inhibition efficiency [1], which was calculated for each column and scavenger individually.

Scavenger Number	Initial NPP [ppm] No NO_2^- Spiking	Final NPP [ppm] No NO_2^- Spiking [3]	Initial NPP [ppm] with NO_2^- Spiking	Final NPP [ppm] with NO_2^- Spiking [3]
Ctrl [2]	1.98	2.37	n.d. [4]	2.67
1	1.89 (−4.2%)	n.d.[4] (−100.0%)	n.d. [4]	1.29 (−51.8%)
2	n.d. [4] (−100.0%)	7.84 (+128.4%)	n.d. [4]	11.18 (+316.2%)
5	n.d. [4] (−100.0%)	2.53 (+8.0%)	n.d. [4]	2.96 (+11.0%)
7	n.d. [4] (−100.0%)	2.50 (+7.6%)	n.d. [4]	2.74 (+1.8%)
15	n.d. [4] (−100.0%)	1.98 (−15.3%)	1.88 (N.A.) [5]	1.07 (−60.2%)
16	2.02 (+2.3%)	1.06 (−55.4%)	n.d. [4]	2.42 (−10.5%)

[1] Inhibition efficiency = 100% × (NPP amount with scavenger − NPP amount in control)/(NPP amount in control). [2] No added scavenger was used as the control for each of the experiments. [3] Values are reported as averages of measurements in two tablets. [4] Values below LOQ of 10 ppb. [5] Inhibition efficiency could not be calculated as the amount of NPP without scavenger was below LOQ.

NMA has been detected in all the tablets with MA × HCl before stress testing, with the highest amount of 97.31 ppm and the lowest in 14.47 ppm (Table 3). More NMA was formed in the tablets spiked with nitrite, which was expected. The increase of *N*-nitrosamine in the non-spiked tablets is probably due to the presence of nitrite in the excipients used [67]. The impact of the scavengers on the *N*-nitrosation was inconclusive. The only scavenger that decreased the amount of NMA relative to the control tablets in both the non-spiked and spiked tablets was sodium ascorbate (**2**). Ascorbic acid (**1**) also slightly decreased the amount of NMA in the non-spiked tablet (by 11.8%) and PABA (**15**) lowered it in the nitrite-spiked tablet by 15.3%. The other three evaluated scavengers (**5**, **7** and **16**, respectively) increased the NMA amount in both the non-spiked and spiked tablets. The impact was much more obvious in the spiked tablets, where the increase compared to the control tablet was 60.2% for maltol (**5**) and 188.1% for propyl gallate (**7**). Further stability tests (data shown in a subsequent section) of NMA were performed at two less stringent stability conditions: 50 °C and 40 °C (moisture excluded by keeping the samples in tightly closed containers).

The amount of formed NPP increased in all but three compositions as the result of stress testing (Table 4). While spiking the tablets with nitrite did not lead to any considerable increase in the *N*-nitrosamine formation before stress testing, it did increase the amount of formed NPP after stress testing compared to the non-spiked tablets. The impact of the nitrite scavengers on the *N*-nitrosation again varied among the different scavengers. Before stress testing, sodium ascorbate, maltol, and propyl gallate (**2**, **5** and **7**, respectively) prevented NPP formation completely. Ascorbic acid (**1**) and L-cysteine (**16**) had no visible impact, but the presence of **15**, on the other hand, increased the amount of NPP in the nitrite-spiked tablet. However, these three scavengers (**1**, **15** and **16**) considerably attenuated the *N*-nitrosation of PP during stress testing, as seen with both the non-spiked and spiked tablets. Interestingly, the other three scavengers (**2**, **5** and **7**) which appeared very effective before stress testing, actually led to increased amounts of formed NPP. Sodium ascorbate (**2**) promoted the *N*-nitrosation of PP × HCl to a significant degree, as the amount of NPP more than doubled in the non-spiked tablets (128.4% increase) and more than quadrupled in the spiked tablets (316.2% increase). Maltol (**5**) and propyl gallate's (**7**) impact on the increase in NPP was only modest (between 1.8 and 11.0%).

4. Discussion

4.1. Nitrite Scavenging

Herein, we present a comprehensive study of an array of nitrite scavengers and their ability to prevent *N*-nitrosamine formation in solution and solid–state experiments. We were able to demonstrate that nitrite scavengers can be used to significantly reduce the amount of *N*-nitrosamines formed from two model APIs, *N*-methylaniline (MA) and *N*-phenylpiperazine (PP), in solution and some also in solid state from the corresponding model API salts MA × HCl and PP × HCl.

In the first part of the study, we have conducted an extensive experimental screening of various potential nitrite scavengers. All but resveratrol (**6**) and *para*-aminobenzoic acid (**15**) are considered as suitable excipients in medicines (included in the FDA's Inactive Ingredients Database). Although the safety profiles of **6** and **15** are still studied, both are safely taken orally as dietary supplements in doses much higher than they would be added to medicines as *N*-nitrosation inhibitors [55,56]. Only one study of such magnitude has been reported so far in the scientific literature [37]. All the evaluated compounds have already been proven to react with the nitrite ion (see Table 2 for references), but we decided to evaluate them again under comparable, pharmaceutically relevant conditions found critical for *N*-nitrosamine formation, to gain a general overview on the reaction kinetics. We have observed that all the scavenging reactions were faster at pH 3.0 compared to pH 5.0. Because pH 3.0 has been identified as the most concerning pH for *N*-nitrosamine formation [20], these results were found to be encouraging. The relative kinetics of nitrite depletion for all the evaluated scavengers was conserved at both pHs. Above pH 6.0, the reaction rates were insignificant. A similar trend was observed when comparing the rates at 50 °C and 20 °C, where a higher temperature significantly increased all the reaction rates.

Most of the evaluated compounds were shown to be at least slightly effective nitrite scavengers, which was in accordance with the literature data on their reactivity. Due to the fact that significant differences in reactivity between the various compounds and scavenger-free control were already observed within 24 h, we based our decision on the selection of the most promising compounds only on the results from this time window. Interestingly, some compounds turned out to be much more effective scavengers than we expected based on the literature data (e.g., maltol (**5**)), while others (BHA (**8**) and BHT (**9**)) were surprisingly inefficient. Amino acids **10**–**13** also showed no scavenging activity, even though some of them were previously shown to be efficient inhibitors of *N*-nitrosation in the solution at pH 3.0, albeit at a very high temperature (60 °C) [39]. Only L-cysteine (**16**) was an efficient proteinogenic amino acid-based scavenger at room temperature, which may indicate that the thiol group is required for activity.

Solubility was another issue that had to be taken into consideration, as seven compounds (**3**, **4**, **6**, **7**, **8**, **9** and **15**) were too lipophilic to be completely soluble at the targeted concentration. Since they were pipetted to the reaction mixture as stock suspensions, their actual concentration was likely much lower than desired (less excess compared to soluble scavengers), although, in theory, it could also be higher if an unproportionally large number of solid particles was added from the stock suspension which would dissolve in the reaction mixture. The optimization of the reaction medium with the co-solvents to achieve the solubility of all the scavengers and thus ensure completely comparable conditions has been unsuccessful (Figure S4). Although this problem could also be solved by adding detergents (e.g., sodium dodecylsulfate [37]), we wanted to avoid a too complex system to avoid additional interactions. As already mentioned in the Introduction, the activity of **1** to prevent *N*-nitrosamine formation may be attenuated by lipids due to the regeneration of nitrosating species with oxygen dissolved in the lipid phase [40]. It is therefore likely that a different set of effective scavengers would be selected if the study was performed in a biphasic system. Due to its relevance for pharmaceutical and in vivo conditions, we decided to primarily choose the best water-soluble compounds and only add two water insoluble scavengers **7** and **15**, which showed very high activity already at partial solubility. The number of selected six compounds (**1**, **2**, **5**, **7**, **15** and **16**) was chosen in order for the

further studies to be as feasible experimentally as possible. It is important to emphasize that other scavengers from the screening panel than the ones selected may also be effective as inhibitors of *N*-nitrosation but could work by a different mechanism than nitrite depletion, such as pH modification [10]. This could also potentially explain the discrepancy between the activity of amino acids **10**–**13** in our study and the study by Nanda [39], where no buffer was used, and amino acids neutralized the studied reaction system diminishing the *N*-nitrosation. Additionally, different chemical and physical factors can govern reactions in the solid state compared to the ones conducted in the solution [68–70]. Therefore, the outcome of the nitrite scavenging in the solid state might be different than the one in the solution, which could provide different results, if the nitrite scavenger screening would be performed directly in the tablets. Due to the study design, these possibilities have not been evaluated, but could be examined in future studies.

4.2. N-Nitrosamine Formation Inhibition in Solution

The results of the scavenger evaluation with model APIs MA and PP may first give us some important insights into the kinetics of *N*-nitrosamine formation. Both MA and PP are secondary amines that were chosen to represent structural motifs in APIs that may be considered most prone for *N*-nitrosation. There is a substantial difference in the reactivity of both compounds in the solution at pH 3.0, since MA was quantitatively transformed to NMA with 1.1 equivalents of nitrite within 30 min, while only approximately 5% of PP reacted to NPP under the same conditions in 24 h (blue curves in Figures 5 and 6). Careful observation of Figure 5 shows that some NMA was observed immediately (less than one minute after mixing all the reaction components together). This shows that alkyl-aryl amines are much more reactive than dialkyl amines, which is in accordance with the observations from the literature and may be explained by the different basicity and thus degree of protonation [57]. The same observation can be made in the solid state, where a significant amount of NMA was formed during tableting (Table 3) and much less NPP under identical conditions (Table 4). The results emphasize that mitigation strategies to prevent the formation of NDSRI are especially important for APIs that contain aniline-type nitrogen in their chemical structure, as these can get *N*-nitrosated immediately after being exposed to nitrite and acidic conditions (e.g., formulation process in acidic conditions).

On the other hand, one can observe that no NMA could be detected in the tablets with MA × HCl, exposed to 75% RH and 50 °C for 1 month, while NPP was present in all the tablets with PP × HCl after stress testing. There was also a slight drop in the NMA concentration in the solution between 0.5 and 2 h (Figure 5). Afterwards, the concentrations remained fairly stable for the remainder of the experiments (up to 8 h). These results indicate that alkyl-aryl *N*-nitrosamines might be less stable than their dialkyl counterparts under the studied conditions. In fact, aromatic *N*-nitrosamines are known to undergo Fischer–Hepp rearrangement under acidic conditions to form *para*-nitrosoarylamines [71]. Since HCl has been acknowledged as the most efficient acid to facilitate this transformation (due to the catalytic activity of Cl^- ion), it is, therefore, possible that the presence of HCl in the tablets facilitated this reaction much more compared to the phosphate buffer medium in the solution experiments. Furthermore, several reports have shown that aryl *N*-nitrosamines are much more prone to reversible denitrosation under acidic conditions compared to alkyl *N*-nitrosamines due to the electron-withdrawing effect of the phenyl group [72–75]. The reaction rates and equilibria are strongly solvent-dependent and the presence of nucleophiles and/or nitrite traps may have a substantial influence [73–75]. In order to evaluate the stability of NMA and to see whether only a longer exposure time and, especially, a higher temperature facilitate its degradation, we performed an additional experiment. Two solid tableting mixtures (nitrite-spiked and non-spiked) containing MA × HCl, already contaminated with NMA, were heated at 50 °C. Sampling after 24 h, 5 days, and 8 days showed a significant drop in the amount of NMA compared to the initial mixtures. Both mixtures were also heated at 40 °C and a large drop in the NMA amount was observed after 8 days as well (Figure 7). We can thus conclude that NMA is indeed

unstable during the conditions of stress testing within 28 days, which explains why it was not present anymore in the tablets with MA × HCl (Table 3).

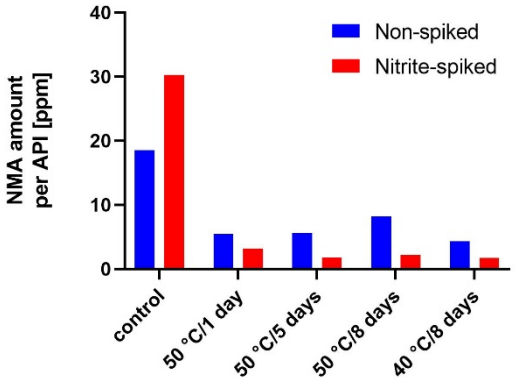

Figure 7. Stability of NMA in solid state at 50 °C and 40 °C. Each measurement was performed once (n = 1).

In order to evaluate the nitrite scavenging action of the selected five compounds during the *N*-nitrosation experiments in the solution, we measured the nitrite concentration with test strips at all the sampling timepoints. The results are presented in the Supplementary material in Figure S5. The initial nitrite concentration of 2.2 mM (101 mg/L) was detected as 80 mg/L due to the upper limitation of the test strips. One may see that the concentration of the nitrite without any scavenger dropped significantly with MA due to the fast *N*-nitrosation kinetics and only 10 mg/L (roughly the excess amount of 0.1 Eq.) remained after 30 min. Interestingly, the nitrite concentration decreased more slowly with **1** present, even though it showed a strong inhibitory effect on NMA formation. Unexpected false positive results were obtained at all timepoints in the reaction mixtures with MA and PABA (**15**), as the test strips showed a strong purple color, which is otherwise indicative for a high nitrite concentration. Such behavior was not observed during the screening experiments and the *N*-nitrosation reactions with PP. The combination of MA/NMA and **15,** thus, likely interacted with the test strip components, which are based on the Griess reaction (diazotation and diazonium salt coupling). However, such a false positive result has not yet been described in the literature. The other three scavengers (**5**, **7** and **16**) led to the complete disappearance of the nitrite after 30 min due to the nitrite being consumed by both the MA and scavengers.

Slower kinetics of the nitrite disappearance were observed in the reaction mixtures with PP (Figure S5). When no scavenger was present, around 80 mg/L of NO_2^- were still detected after 2 h and, even after 24 h, some traces were still present. Interestingly, the amount of NPP did not increase any more after approximately 2 h either (Figure 5), even though enough nitrite was still present. This indicates that other species in the reaction mixture prevented further *N*-nitrosation (e.g., another reaction side product) or that a dynamic equilibrium between the NPP formation and degradation was established. The slow decline of the NPP concentration after 2 h (when there was less nitrite present and NPP degradation started to prevail over its formation) may contribute some evidence to this hypothesis. All scavengers did substantially increase the rate of the nitrite disappearance, as no more nitrite was detected in any of the mixtures after 8 h. Scavengers **7** and **16** appeared to have been the most efficient, as only traces of NO_2^- were measured after 1 h. The rate of the nitrite depletion, therefore, does not correlate with the *N*-nitrosation inhibitory efficiency, as the scavengers that inhibited NMA and NPP formation to the greatest extent were not the ones which increased the rate of the nitrite depletion the most.

4.3. N-Nitrosation Inhibition Activity Comparison of Selected Scavengers in Solid State and Solution

Ascorbic acid (**1**) was the most effective nitrite scavenger overall, as it almost completely prevented the formation of NMA and NPP in the solution and significantly decreased the amount of NPP in the tablets (Figures 5 and 6 and Table 4). This is in accordance

with the literature data [37,39,59], as already presented in the Introduction section. Interestingly, **1** was not as efficient as expected in preventing NMA formation (Table 3) during tableting. One possible reason for this could be its relatively high acidity, which led to a proton transfer to the nitrite (thus activating it) prevailing over its reduction. The better activity of its non-acidic analogue sodium ascorbate **2** in the same setting supports this hypothesis. This study thus provides further proof that **1** could be utilized in medicinal products to prevent *N*-nitrosation but may not act as a "magic bullet" that would be effective under all circumstances and should be evaluated on a case-by-case basis. Its mechanism of action as a nitrite scavenger is well-known, including the structure of the products formed (dehydroascorbic acid and nitric oxide) [35].

Para-aminobenzoic acid (**15**) was also a very efficient inhibitor of NMA and NPP formation. It was even more effective in the solution than **1** (Figures 5 and 6), but its activity in the solid state was slightly lower than **1** in the tablets with PP × HCl (Table 4) and it also increased the amount of NMA formed from MA × HCl (Table 3). *Para*-hydroxybenzoic acid has been identified as the product of diazotation and diazonium substitutions reactions between **15** and nitrite ion in acidic medium. Due to the fact that **15** has so far only been confirmed as a nitrite scavenger in foodstuff [52], this study represents the first evidence that it may also be used as an *N*-nitrosation inhibitor in medicines. However, **15** is not included in the FDA's Inactive Ingredients Database, although it occurs naturally in human bodies as a metabolite [76]. It is important to note that **15** was one of the less soluble scavengers (along with **7**), which means that it is likely that its concentration (and, consequently, excess) in the solution was actually lower than the concentration of the other scavengers. One may conclude that its activity in the solution might be relatively even higher, if the desired concentration could be reached.

The activity of L-cysteine (**16**) in the solution against *N*-nitrosamine formation was comparable to ascorbic acid's (Figures 5 and 6), but it was less effective in preventing NPP formation (Table 4) and increased the amount of NMA (Table 3) in the tablets. Its effectiveness for the prevention of *N*-nitrosation has also already been established in the literature and it presumably acts via the reduction of nitrite to nitric oxide as well [37]. This reaction is likely pH-dependent, occurring more rapidly at a higher pH. The higher inhibitory activity of **16** in the buffered solutions compared to the more neutral environment in the tablets is, thus, well in agreement with the proposed reaction pathways.

Propyl gallate (**7**) was an efficient inhibitor of NPP formation in the solution (Figure 5) but did not have any significant impact in the tablets (Table 4). It was less effective against NMA formation in the solution (Figure 5), and it increased the amount of NMA in the tablets (Table 3). The dual behavior of phenolic acids has been noted in the literature already [37], where at least two *ortho*-positioned hydroxy groups on an aromatic ring facilitate nitrite reduction to NO through oxidation to an *ortho*-quinone. Aromatic ring *C*-nitrosation is also a possibility; however, it may further lead to the transnitrosation of the nitroso group to an amino functionality, thus catalyzing the *N*-nitrosation. The latter effect is more pronounced at a pH above 4, which may explain why it occurred in the tablets with a mildly acidic PP × HCl rather than a more acidic MA × HCl. Like compound **15**, **7** was insoluble at the desired stoichiometry, so less of an excess than desired was used in the solution experiments. It is known for phenolic acids that a higher ratio of scavenger: nitrite may provide a better inhibitory effect, which could explain why **7** was less effective in the solution than **1** and **16**.

Maltol (**5**) was the weakest inhibitor of NMA and NPP formation in the solution (Figures 5 and 6). Its activity in the tablets was very similar to **7**, having none in the tablets with PP × HCl (Table 4) and slightly increasing the amount of NMA in the tablets with MA × HCl (Table 3). Due to the fact that the literature data of its activity for nitrite scavenging was scarce [44] and only modest activity was reported, its high activity in our screening system (Figure 2) was rather unexpected in the first place. Its inhibitory effect on *N*-nitrosamine formation in cosmetic products has been reported in the non-scientific literature [77] where it significantly decreased the amount of NDMA, NDEA, NMOR and

NDELA in nail polish. Our observations, however, do not show any significant benefits of adding maltol to medicinal products, since much more efficient alternatives are available. The lack of an unsaturated vicinal diol moiety (present in **1**, phenolic acids, and other flavonoids) has been suggested as the main reason for its low activity [44].

Sodium ascorbate (**2**) was evaluated only in the solid state as it gets protonated in the solution to form **1** at pH 3.0 (ascorbic acid's pK_a is 4.29 [35]). While it modestly decreased the amount of NMA after tableting (Table 3), it strongly catalyzed the *N*-nitrosation of PP × HCl to NPP (Table 4). Such behavior has not yet been reported for this compound, as it efficiently inhibited the *N*-nitrosation in a comparable study [39]. However, as discussed above with **16**, the promotion of *N*-nitrosamine formation has already been observed for certain compounds that were otherwise effective inhibitors at different conditions (e.g., glutathione) [37].

One observation is also that **1**, **15** and **16** appear to decrease the amount of NPP during stress testing when it was already present initially (Table 4). This may be explained by the fact that only one tablet per composition was analyzed prior to stress testing and two tablets after testing, which means that the values do not represent a statistically significant population of tablets. However, based on the instability of NMA, one could also conclude that NPP may get degraded slowly with time, which becomes obvious when it is present in sufficient amounts from the beginning. Analysis of a larger number of samples and more frequent sampling could answer this question.

It should also be noted that there was a significant change in color observed after the stress test with some scavengers (Figure S6). This was most pronounced with scavengers **1**, **2** and **15** and is due to the chemical degradation of the scavenger. Ascorbic acid (1) is known to be unstable and discolors over time [78]. Thus, including it as a scavenger would likely require strict storage conditions (e.g., protected from humidity, inert atmosphere).

5. Conclusions

In this study, we have presented a comprehensive two-step approach into identifying structurally diverse nitrite scavengers as potential inhibitors of the *N*-nitrosation of secondary amines in solution and solid state. Initially, we have conducted a broad and systematic nitrite scavenging screening with nineteen compounds that were able to deplete the nitrite in a solution. Subsequently, the study was complemented by solution and solid-state experiments on the *N*-nitrosation inhibition using model dialkylamine and alkylarylamine substrates and the six best performing nitrite scavengers identified in the initial screening. The study revealed that, among the tested nineteen nitrite scavengers, three groups of compounds demonstrated superior nitrite scavenging activity: antioxidants (vitamin C derivatives and propyl gallate), 3-hydroxy-4-pyrone derivative (maltol), and amino acids (*para*-aminobenzoic acid and L-cysteine). We reveal for the first time that PABA acts as an efficient *N*-nitrosamine formation inhibitor in pharmaceutical dosage forms and has comparable activity to ascorbic acid and L-cysteine. This study demonstrates that a careful selection of a nitrite scavenger, such as vitamin C, PABA, or L-cysteine, should be done based on the structure of the amine (e.g., dialkylamine and alkyl–arylamine), as nitrite scavenging does not always translate to the inhibition of *N*-nitrosamine formation [37]. The chemical compatibility of the scavenger with the API and excipients, type of dosage form, and route of administration should also be taken into consideration.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/pr10112428/s1, Figure S1: The result of the screening experiment with the 19 shortlisted scavengers (20 °C, pH 5.0); Figure S2: The result of the screening experiment with the 19 shortlisted scavengers (50 °C, pH 3.0); Figure S3: The result of the screening experiment with the 19 shortlisted scavengers (50 °C, pH 5.0); Figure S4: The result of the screening experiment with the 8 water-insoluble scavengers in 75% *v*/*v* DMSO in pH 3.0 or pH 5.0; Figure S5: NO_2^- concentrations during experiments for evaluation of *N*-nitrosation inhibitory activity of selected nitrite scavengers; Figure S6: Tablets after 28-day stress testing at 50 °C and 75% RH; Figure S7: Representative LC chromatograms for the determination of NMA; Figure S8: Representative LC chromatograms for the determination of NPP.

Author Contributions: Conceptualization, M.H., M.T., M.K. and Z.Č.; methodology, M.H., M.T., K.N., M.Ž., Z.K., M.K. and Z.Č.; data curation, M.H., M.T., K.N., M.Ž. and Z.K.; funding acquisition, Z.Č.; formal analysis M.H., M.T., K.N., M.Ž. and Z.K.; investigation, M.H., M.T., K.N., M.Ž. and Z.K.; project administration, Z.Č.; resources, Z.Č.; supervision, Z.Č.; validation, M.H., M.T., K.N., M.Ž. and Z.K.; visualization, M.H., M.T. and Z.Č.; writing—original draft, M.H., M.T., K.N., Z.K. and Z.Č.; writing—review and editing, M.H., M.T., K.N., M.Ž., Z.K.; M.K. and Z.Č. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by Lek Pharmaceuticals d.d., a Sandoz company. A part of this research was conducted under the Novartis Genesis Labs project (Cohort Three).

Data Availability Statement: Not applicable.

Acknowledgments: The authors would gratefully like to acknowledge S. Akula, J. Cluzeau, N. Golob, K. Gosak, R. Grahek, J. Hren, T. Mahapatra, S. Pirc, D. Šterk, D. Teslić, and B. Zupančič from Sandoz and U. Onken from Novartis for the productive discussions during the selection of potential nitrite scavengers.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

References

1. Fritzsche, M.; Blom, G.; Keitel, J.; Goettsche, A.; Seegel, M.; Leicht, S.; Guessregen, B.; Hickert, S.; Reifenberg, P.; Cimelli, A.; et al. NDMA Analytics in Metformin Products: Comparison of Methods and Pitfalls. *Eur. J. Pharm. Sci.* **2022**, *168*, 106026. [CrossRef] [PubMed]
2. Rudolph, U.M.; Enners, S.; Kieble, M.; Mahfoud, F.; Böhm, M.; Laufs, U.; Schulz, M. Impact of Angiotensin Receptor Blocker Product Recalls on Antihypertensive Prescribing in Germany. *J. Hum. Hypertens.* **2021**, *35*, 903–911. [CrossRef] [PubMed]
3. Ruepp, R.; Frötschl, R.; Bream, R.; Filancia, M.; Girard, T.; Spinei, A.; Weise, M.; Whomsley, R. The EU Response to the Presence of Nitrosamine Impurities in Medicines. *Front. Med.* **2021**, *8*, 4–6. [CrossRef] [PubMed]
4. Ray, A.; Atal, S.; Sadasivam, B. Understanding the Molecular-Pharmaceutical Basis of Sartan Recalls Focusing on Valsartan. *Glob. Cardiol. Sci. Pract.* **2020**, *25*. [CrossRef] [PubMed]
5. Byrd, J.B.; Chertow, G.M.; Bhalla, V. Hypertension Hot Potato-Anatomy of the Angiotensis Receptor Blocker Recalls. *N. Engl. J. Med.* **2019**, *380*, 1589–1591. [CrossRef]
6. Gunasekaran, P.M.; Chertow, G.M.; Bhalla, V.; Byrd, J.B. Current Status of Angiotensin Receptor Blocker Recalls. *Hypertension* **2019**, *74*, 1275–1278. [CrossRef]
7. European Medicines Agency. EMA/369136/2020–Nitrosamine Impurities in Human Medicinal Products. Available online: https://www.ema.europa.eu/en/documents/referral/nitrosamines-emea-h-a53-1490-assessment-report_en.pdf (accessed on 18 October 2022).
8. Bharate, S.S. Critical Analysis of Drug Product Recalls Due to Nitrosamine Impurities. *J. Med. Chem.* **2021**, *64*, 2923–2936. [CrossRef]
9. Schmidtsdorff, S.; Neumann, J.; Schmidt, A.H.; Parr, M.K. Risk Assessment for Nitrosated Pharmaceuticals: A Future Perspective in Drug Development. *Arch. Pharm.* **2022**, *355*, e2100435. [CrossRef]
10. U.S. Food & Drug Administration. Control of Nitrosamine Impurities in Human Drugs. Available online: https://www.fda.gov/media/141720/download (accessed on 18 October 2022).
11. Montesano, R. Alkylation of DNA and Tissue Specificity in Nitrosamine Carcinogenesis. *J. Supramol. Struct. Cell. Biochem.* **1981**, *17*, 259–273. [CrossRef]
12. Tricker, A.R.; Preussmann, R. Carcinogenic N-Nitrosamines in the Diet: Occurrence, Formation, Mechanisms and Carcinogenic Potential. *Mutat. Res.* **1991**, *259*, 277–289. [CrossRef]
13. Kroeger-Koepke, M.B.; Koepke, S.R.; McClusky, G.A.; Magee, P.N.; Michejda, C.J. α-Hydroxylation Pathway in the in Vitro Metabolism of Carcinogenic Nitrosamines: N-Nitrosodimethylamine and N-Nitroso-N-Methylaniline. *Proc. Natl. Acad. Sci. USA* **1981**, *78*, 6489–6493. [CrossRef] [PubMed]
14. Guengerich, F.P. Oxidation of Toxic and Carcinogenic Chemicals by Human Cytochrome P-450 Enzymes. *Chem. Res. Toxicol.* **1991**, *4*, 391–407. [CrossRef] [PubMed]
15. Li, Y.; Hecht, S.S. Metabolic Activation and DNA Interactions of Carcinogenic N-Nitrosamines to Which Humans Are Commonly Exposed. *Int. J. Mol. Sci.* **2022**, *23*, 4559. [CrossRef] [PubMed]
16. Li, Y.; Hecht, S.S. Metabolism and DNA Adduct Formation of Tobacco-Specific N-Nitrosamines. *Int. J. Mol. Sci.* **2022**, *23*, 5109. [CrossRef] [PubMed]
17. Jakszyn, P.; González, C.A. Nitrosamine and Related Food Intake and Gastric and Oesophageal Cancer Risk: A Systematic Review of the Epidemiological Evidence. *World J. Gastroenterol.* **2006**, *12*, 4296–4303. [CrossRef]
18. International Agency for Research on Cancer. List of Classifications–IARC Monographs on the Identification of Carcinogenic Hazards to Humans. Available online: https://monographs.iarc.who.int/list-of-classifications/ (accessed on 11 November 2022).

19. Nawrocki, J.; Andrzejewski, P. Nitrosamines and Water. *J. Hazard. Mat.* **2011**, *189*, 1–18. [CrossRef]
20. Ashworth, I.W.; Dirat, O.; Teasdale, A.; Whiting, M. Potential for the Formation of N-Nitrosamines during the Manufacture of Active Pharmaceutical Ingredients: An Assessment of the Risk Posed by Trace Nitrite in Water. *Org. Process Res. Dev.* **2020**, *24*, 1629–1646. [CrossRef]
21. Langford, V.S.; Gray, J.D.C.; Maclagan, R.G.A.R.; Milligan, D.B.; McEwan, M.J. Real-Time Measurements of Nitrosamines in Air. *Int. J. Mass Spectrom.* **2015**, *377*, 490–495. [CrossRef]
22. Choi, N.R.; Ahn, Y.G.; Lee, J.Y.; Kim, E.; Kim, S.; Park, S.M.; Song, I.H.; Kim, Y.P. Winter-Time Particulate Nitrosamines and Nitramines in the Atmosphere at Seoul, South Korea. *Atmos. Environ.* **2020**, *237*, 117582. [CrossRef]
23. Pancholy, S.K. Formation of Carcinogenic Nitrosamines in Soils. *Soil Biol. Biochem.* **1978**, *10*, 27–32. [CrossRef]
24. Yoneyama, T. Detection of N-Nitrosodimethylamine in Soils Amended with Sludges. *Soil Sci. Plant Nutr.* **1981**, *27*, 249–253. [CrossRef]
25. Khan, S.U.; Young, J.C. N-Nitrosamine Formation in Soil from the Herbicide Glyphosate. *J. Agric. Food Chem.* **1977**, *25*, 1430–1432. [CrossRef] [PubMed]
26. Hecht, S.S. Biochemistry, Biology, and Carcinogenicity of Tobacco-Specific N-Nitrosamines. *Chem. Res. Toxicol.* **1998**, *11*, 559–603. [CrossRef] [PubMed]
27. Altkofer, W.; Braune, S.; Ellendt, K.; Kettl-Grömminger, M.; Steiner, G. Migration of Nitrosamines from Rubber Products—Are Balloons and Condoms Harmful to the Human Health? *Mol. Nutr. Food Res.* **2005**, *49*, 235–238. [CrossRef] [PubMed]
28. Challis, B.C.; Trew, D.F.; Guthrie, W.G.; Roper, D.V. Reduction of Nitrosamines in Cosmetic Products. *Int. J. Cosmet. Sci.* **1995**, *17*, 119–131. [CrossRef]
29. Tuesuwan, B.; Vongsutilers, V. Nitrosamine Contamination in Pharmaceuticals: Threat, Impact, and Control. *J. Pharm. Sci.* **2021**, *110*, 3118–3128. [CrossRef] [PubMed]
30. Parr, M.K.; Joseph, J.F. NDMA Impurity in Valsartan and Other Pharmaceutical Products: Analytical Methods for the Determination of N-Nitrosamines. *J. Pharm. Biomed. Anal.* **2019**, *164*, 536–549. [CrossRef]
31. López-Rodríguez, R.; McManus, J.A.; Murphy, N.S.; Ott, M.A.; Burns, M.J. Pathways for N-Nitroso Compound Formation: Secondary Amines and Beyond. *Org. Process Res. Dev.* **2020**, *24*, 1558–1585. [CrossRef]
32. Itoh, T.; Matsuya, Y.; Maeta, H.; Miyazaki, M.; Nagata, K.; Ohsawa, A. Effect of Oxygen on the Reaction of Secondary Amines with Nitric Oxide. *Chem. Pharm. Bull.* **1999**, *47*, 133–135. [CrossRef]
33. Wu, Y.; Levons, J.; Narang, A.S.; Raghavan, K.; Rao, V.M. Reactive Impurities in Excipients: Profiling, Identification and Mitigation of Drug-Excipient Incompatibility. *AAPS Pharm. Sci. Tech.* **2011**, *12*, 1248–1263. [CrossRef]
34. Schlingemann, J.; Boucley, C.; Hickert, S.; Bourasseau, L.; Walker, M.; Celdran, C.; Chemarin, T.; Pegues, C.; Fritzsche, M.; Keitel, J.; et al. Avoiding N-Nitrosodimethylamine Formation in Metformin Pharmaceuticals by Limiting Dimethylamine and Nitrite. *Int. J. Pharm.* **2022**, *620*, 121740. [CrossRef] [PubMed]
35. Mirvish, S.S.; Wallcave, L.; Eagen, M.; Shubik, P. Ascorbate-Nitrite Reaction: Possible Means of Blocking the Formation of Carcinogenic N-Nitroso Compounds. *Science* **1972**, *177*, 65–68. [CrossRef] [PubMed]
36. Tannenbaum, S.R. Preventive Action of Vitamin C on Nitrosamine Formation. *Int. J. Vitam. Nutr. Res.* **1989**, *30*, 109–113.
37. Rundlöf, T.; Olsson, E.; Wiernik, A.; Back, S.; Aune, M.; Johansson, L.; Wahlberg, I. Potential Nitrite Scavengers as Inhibitors of the Formation of N-Nitrosamines in Solution and Tobacco Matrix Systems. *J. Agric. Food Chem.* **2000**, *48*, 4381–4388. [CrossRef]
38. Lim, D.S.; Lim, S.K.; Kim, M.K.; Kwon, Y.C.; Roh, T.H.; Choi, S.M.; Yoon, S.; Kim, H.S.; Lee, B.M. Formation and Inhibition of N-Nitrosodiethanolamine in Cosmetics under pH, Temperature, and Fluorescent, Ultraviolet, and Visual Light. *J. Toxicol. Environ. Health Part A* **2018**, *81*, 241–253. [CrossRef]
39. Nanda, K.K.; Tignor, S.; Clancy, J.; Marota, M.J.; Allain, L.R.; D'Addio, S.M. Inhibition of N-Nitrosamine Formation in Drug Products: A Model Study. *J. Pharm. Sci.* **2021**, *110*, 3773–3775. [CrossRef]
40. Combet, E.; Paterson, S.; Iijima, K.; Winter, J.; Mullen, W.; Crozier, A.; Preston, T.; McColl, K.E.L. Fat Transforms Ascorbic Acid from Inhibiting to Promoting Acid-Catalysed N-Nitrosation. *Gut* **2007**, *56*, 1678–1684. [CrossRef]
41. Combet, E.; el Mesmari, A.; Preston, T.; Crozier, A.; McColl, K.E.L. Dietary Phenolic Acids and Ascorbic Acid: Influence on Acid-Catalyzed Nitrosative Chemistry in the Presence and Absence of Lipids. *Free Radical Biol. Med.* **2010**, *48*, 763–771. [CrossRef]
42. Sen, N.P.; Donaldson, B.; Seaman, S.; Iyengar, J.R.; Miles, W.F. Inhibition of Nitrosamine Formation in Fried Bacon by Propyl Gallate and L-Ascorbyl Palmitate. *J. Agric. Food Chem.* **1976**, *24*, 397–401. [CrossRef]
43. Herrmann, S.S.; Granby, K.; Duedahl-Olesen, L. Formation and Mitigation of N-Nitrosamines in Nitrite Preserved Cooked Sausages. *Food Chem.* **2015**, *174*, 516–526. [CrossRef]
44. Choi, J.S.; Park, S.H.; Choi, J.H. Nitrite Scavenging Effect by Flavonoids and Its Structure-Effect Relationship. *Arch. Pharm. Res.* **1989**, *12*, 26–33. [CrossRef]
45. Lu, Y.; Dong, Y.; Li, X.; He, Q. The Nitrite-Scavenging Properties of Catechol, Resorcinol, and Hydroquinone: A Comparative Study on Their Nitration and Nitrosation Reactions. *J. Food Sci.* **2016**, *81*, C2692–C2696. [CrossRef] [PubMed]
46. Astill, B.D.; Mulligan, L.T. Phenolic Antioxidans and the Inhibition of Hepatotoxicity from N-Dimethylnitrosamine Formed in Situ in the Rat Stomach. *Food Cosmet. Toxicol.* **1977**, *15*, 167–171. [CrossRef]
47. Hughes, M.N. Kinetic Study of the Reaction between Nitrous Acid and Sulphamic Acid. *J. Chem. Soc. A* **1967**, 902–905. [CrossRef]
48. Fitzpatrick, J.; Meyer, T.A.; O'Neill, M.E.; Williams, D.L.H. Comparison of the Reactivity of Nine Nitrous Acid Scavengers. *J. Chem. Soc. Perkin Trans. 2* **1984**, *1984*, 927–932. [CrossRef]

49. Stedman, G. Mechanism of the Azide-Nitrite Reaction. Part IV. *J. Chem. Soc.* **1960**, 1702–1709. [CrossRef]
50. Hughes, M.N.; Stedman, G. Kinetics and Mechanism of the Reaction between Nitrous Acid and Hydroxylamine. Part I. *J. Chem. Soc.* **1963**, 2824–2830. [CrossRef]
51. Bryant, T.; Williams, D.L.H. Nitrosation of Ammonia. *J. Chem. Soc. Perkin Trans. 2* **1988**, *1988*, 97–99. [CrossRef]
52. Kako, Y.; Toyoda, Y.; Hatanaka, Y.; Suwa, Y.; Nukaya, H.; Nagao, M. Inhibition of mutagenesis by p-aminobenzoic acid as a nitrite scavenger. *Mutat. Res.* **1992**, *282*, 119–125. [CrossRef]
53. Nguyen, D.A.; Iwaniw, M.A.; Fogler, H.S. Kinetics and Mechanism of the Reaction between Ammonium and Nitrite Ions: Experimental and Theoretical Studies. *Chem. Eng. Sci.* **2003**, *58*, 4351–4362. [CrossRef]
54. Kato, T.; Kikugawa, K. Proteins and Amino Acids as Scavengers of Nitrite: Inhibitory Effect on the Formation of Nitrosodimethylamine and Diazoquinone. *Food Chem. Toxicol.* **1992**, *30*, 617–626. [CrossRef]
55. Salehi, B.; Mishra, A.P.; Nigam, M.; Sener, B.; Kilic, M.; Sharifi-Rad, M.; Fokou, P.V.T.; Martins, N.; Sharifi-Rad, J. Resveratrol: A Double-Edged Sword in Health Benefits. *Biomedicines* **2018**, *6*, 91. [CrossRef] [PubMed]
56. Maki, T.; Takeda, K. Benzoic Acid and Derivatives. In *Ullmann's Encyclopedia of Industrial Chemistry*; Wiley-VCH Verlag GmbH & Co. KGaA: Hoboken, NJ, USA, 2000; Volume 5, pp. 329–342.
57. Chaudhary, P.; Gupta, S.; Muniyappan, N.; Sabiah, S.; Kandasamy, J. An Efficient Synthesis of N-Nitrosamines under Solvent, Metal and Acid Free Conditions Using Tert -Butyl Nitrite. *Green Chem.* **2016**, *18*, 2323. [CrossRef]
58. Bugarin, A.; Jones, K.D.; Connell, B.T. Efficient, Direct α-Methylenation of Carbonyls Mediated by Diisopropylammonium Trifluoroacetate. *Chem. Commun.* **2010**, *46*, 1715–1717. [CrossRef]
59. Erkekoglu, P.; Baydar, T. Evaluation of the Protective Effect of Ascorbic Acid on Nitrite- and Nitrosamine-Induced Cytotoxicity and Genotoxicity in Human Hepatoma Line. *Toxicol. Mech. Methods* **2010**, *20*, 45–52. [CrossRef]
60. European Federation of Pharmaceutical Industries and Associations. N-Nitrosamine Impurities in Biological Medicinal Products. Available online: https://efpia.eu/media/580595/n-nitrosamine-impurities-in-biological-medicinal-products.pdf (accessed on 18 October 2022).
61. Mergens, W.J.; Newmark, H.L. Antioxidants as Blocking Agents against Nitrosamine Formation. In *Autoxidation in Food and Biological Systems*; Simic, M.G., Karel, M., Eds.; Springer: New York, NY, USA, 1980; pp. 387–403.
62. Digenis, G.A.; Issidorides, C.H. Some Biochemical Aspects of N-Nitroso Compounds. *Bioorg. Chem.* **1979**, *8*, 97–137. [CrossRef]
63. Delmas, D.; Lan, A.; Colin, D.; Jannin, B.; Lançon, A.; Latruffe, N. Resveratrol as a Chemopreventive Agent: A Promising Molecule for Fighting Cancer. *Curr. Drug Targets* **2006**, *7*, 423–442. [CrossRef]
64. Mahal, H.S.; Mukherjee, T. Scavenging of Reactive Oxygen Radicals by Resveratrol: Antioxidant Effect. *Res. Chem. Intermed.* **2006**, *32*, 59–71. [CrossRef]
65. Botterweck, A.A.M.; Verhagen, H.; Goldbohm, R.A.; Kleinjans, J.; van den Brandt, P.A. Intake of Butylated Hydroxyanisole and Butylated Hydroxytoluene and Stomach Cancer Risk: Results from Analyses in the Netherlands Cohort Study. *Food Chem. Toxicol.* **2000**, *38*, 599–605. [CrossRef]
66. Braida, W.; Ong, S.K. Decomposition of Nitrite under Various PH and Aeration Conditions. *Water Air Soil Pollut.* **2000**, *118*, 13–26. [CrossRef]
67. Boetzel, R.; Schlingemann, J.; Hickert, S.; Korn, C.; Kocks, G.; Luck, B.; Blom, G.; Harrison, M.; François, M.; Allain, L.; et al. A Nitrite Excipient Database: A Useful Tool to Support N-Nitrosamine Risk Assessments for Drug Products. *J. Pharm. Sci.* 2022, *in press*. [CrossRef]
68. Byrn, S.R. Mechanisms of Solid-state Reactions of Drugs. *J. Pharm. Sci.* **1976**, *65*, 1–22. [CrossRef] [PubMed]
69. Singh, N.B.; Singh, R.J.; Singh, N.P. Organic Solid State Reactivity. *Tetrahedron* **1994**, *50*, 6441–6493. [CrossRef]
70. Ropp, R.C. Chapter 4—Mechanisms and Reactions in the Solid State. In *Solid State Chemistry*; Ropp, R.C., Ed.; Elsevier Science: New York, NY, USA, 2003; pp. 129–189, ISBN 9780444514363.
71. Wang, Z. Fischer-Hepp Rearrangement. In *Comprehensive Organic Name Reactions and Reagents*; John Wiley & Sons, Inc: Hoboken, NJ, USA, 2010; pp. 1091–1094, ISBN 9780471704508.
72. Gowenlock, B.G.; Pfab, J.; Young, V.M. Photolysis of Some N-Nitroso- and N-Nitro-Anilines in Solution. *J. Chem. Soc. Perkin Trans. 2* **1997**, *1997*, 915–919. [CrossRef]
73. Dix, L.R.; Oh, S.M.N.Y.F.; Williams, D.L.H. Denitrosation of Nitrosamines-a Quantitative Study. Reactions of N-Methyl-N-Nitrosoaniline, N-Nitrosoproline, Dimethyl Nitrosamine and N-Nitrososarcosine. *J. Chem. Soc. Perkin Trans. 2* **1991**, *1991*, 1099–1104. [CrossRef]
74. Al-Kaabi, S.S.; Hallett, G.; Meyer, T.A.; Williams, D.L.H. Unusual Rate-Limiting Proton Transfer in the Acid-Catalysed Reactions of N-Nitroso Compounds. *J. Chem. Soc. Perkin Trans. 2* **1984**, *1984*, 1803–1807. [CrossRef]
75. Johal, S.S.; Williams, D.L.H.; Buncel, E. Kinetics and Mechanism of the Denitrosation of Nitrosamines in Ethanol. *J. Chem. Soc. Perkin Trans. 2* **1980**, *1980*, 165–169. [CrossRef]
76. Rossi, M.; Amaretti, A.; Raimondi, S. Folate Production by Probiotic Bacteria. *Nutrients* **2011**, *3*, 118–134. [CrossRef]
77. Cisneros, R.; Derosier, F. Maltol: To the End of Nitrosamines in Nail Polish? Available online: https://www.premiumbeautynews.com/en/maltol-to-the-end-of-nitrosamines,19250 (accessed on 18 October 2022).
78. Jutkus, R.A.L.; Li, N.; Taylor, L.S.; Mauer, L.J. Effect of Temperature and Initial Moisture Content on the Chemical Stability and Color Change of Various Forms of Vitamin C. *Int. J. Food Prop.* **2015**, *18*, 862–879. [CrossRef]