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Appendix 2 to Questions and answers for marketing authorisation holders/applicants on the CHMP Opinion for the Article 5(3) of Regulation (EC) No 726/2004 referral on nitrosamine impurities in human medicinal products

Carcinogenic Potency Categorisation Approach for N-nitrosamines

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This document describes an approach for assigning an *N*-nitrosamine impurity (including nitrosamine drug substance-related impurities [NDSRIs]) to a predicted carcinogenic potency category, with a corresponding acceptable intake (AI) limit, based on an assessment of activating or deactivating structural features present in the molecule. In the context of this document, activating or deactivating features are defined as molecular substructures that are associated with an increase or decrease, respectively, in carcinogenic potency.

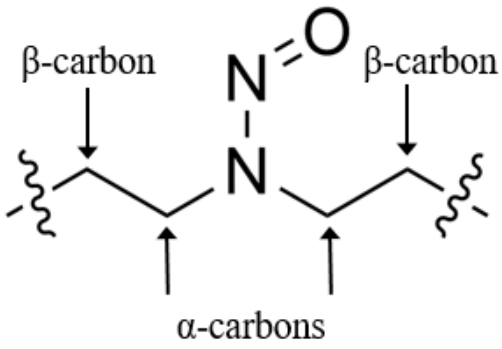
The Carcinogenic Potency Categorisation Approach is based on structure-activity relationship (SAR) concepts described in recent scientific publications for *N*-nitrosamine compounds[1] and also used a set of 84 *N*-nitrosamines with either rat TD50 values from the Carcinogenic Potency Database (CPDB) and/or the Lhasa Carcinogenicity Database (LCDB)[2], relative potency classifications as defined by Rao et al. (1979)[3], and/or AI limits based on previously-conducted surrogate analyses[4]. The approach assumes that the α-hydroxylation mechanism of metabolic activation[5] is responsible for the mutagenic and highly potent carcinogenic response observed for many *N*-nitrosamines. Structural features that directly increase or decrease the favourability of the activation mechanism – or that increase the clearance of the nitrosamine by other biological pathways – are expected to have a corresponding effect on carcinogenic potency. Therefore, a prediction of the mutagenic potential and carcinogenic potency of an *N*-nitrosamine can be generated based on its structural features.

It is recognised that the science is evolving in the prediction of mutagenic potential and carcinogenic potency based on SAR concepts. Therefore, the predicted Carcinogenic Potency Categorisation Approach described in this document is a conservative approach that represents the best available science at this time and is expected to be further refined and expanded as new data become available. This may include refinement of the AI limits associated with predicted carcinogenic potency categories and changes to the structural features and their associated activating and deactivating feature scores.

The Carcinogenic Potency Categorisation Approach applies to *N*-nitrosamines bearing a carbon atom on both sides of the *N*-nitroso group, and where the carbon is not directly double bonded to a heteroatom (i.e., *N*-nitrosamides, *N*-nitrosoureas, *N*-nitrosoguanidines and other related structures are excluded). Additionally, the potency categorisation approach does not apply to *N*-nitrosamines where the *N*-nitroso group is attached to a nitrogen within a hetero aromatic ring (e.g., nitrosated indole). For *N*-nitrosamines containing two *N*-nitroso groups, the group with the highest predicted carcinogenic potency (i.e., the group with the lowest numerical potency category) defines the AI for the entire molecule[6]. The α- and β-carbons are defined relative to the *N*-nitroso group, as illustrated in Figure 1.

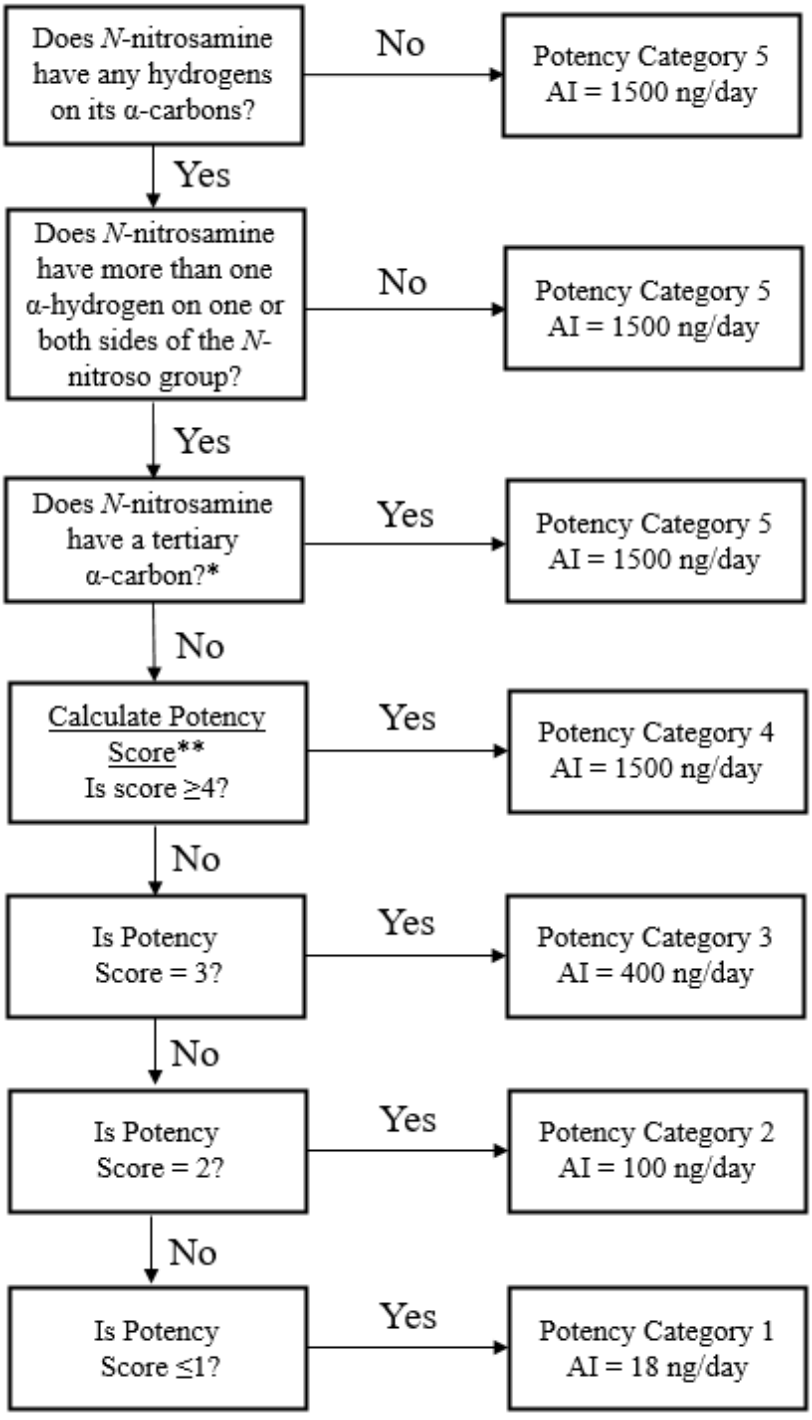
[1] For example, see Cross KP and Ponting DJ, 2021. Developing Structure-Activity Relationships for *N*-Nitrosamine Activity, Comput Toxicol, 20:100186; Thomas R, Tennant RE, Oliveira AAF, and Ponting DJ, 2022. What Makes a Potent Nitrosamine? Statistical Validation of Expert-Derived Structure-Activity Relationships, Chem Res Toxicol, 35:1997–2013; and Ponting DJ, Dobo KL, Kenyon MO, and Kalgutkar AS, 2022. Strategies for Assessing Acceptable Intakes for Novel *N*-Nitrosamines Derived From Active Pharmaceutical Ingredients, J Med Chem, 65:15584–15607.
[2] See Lhasa Carcinogenicity Database at https://carcdb.lhasalimited.org/.
[3] Rao TK, Young JA, Lijinsky W and Epler JL, 1979. Mutagenicity of Aliphatic Nitrosamines in Salmonella typhimurium, Mutat Res, 66:1-7.
[4] Questions and answers for marketing authorisation holders / applicants on the CHMP opinion for the Article 5(3) referral.
[5] Li Y, Hecht SS, 2022. Metabolic Activation and DNA Interactions of Carcinogenic N-Nitrosamines to Which Humans Are Commonly Exposed, Int J Mol Sci, 23:4559.
[6] For *N*-nitrosamines containing more than two *N*-nitroso groups, the applicant or manufacturer should contact the applicable drug regulatory authority for further guidance.

Figure 1. Structural Representation of α- and β-carbons on an *N*-nitrosamine



The process for predicting the appropriate carcinogenic potency category is described in Figure 2. Table 1 summarises the five predicted carcinogenic potency categories and their associated AI limits. Supporting tables to calculate the Potency Score referenced in Figure 2 are in Annex A and example calculations are presented in Annex B.

Figure 2. Flowchart to Predict the Potency Category of an N-nitrosamine



* A tertiary α-carbon is defined as an α-carbon atom in an sp^3 hybridisation state, bonded to three other carbon atoms.

** To calculate Potency Score, see Annex A.

Table 1. The Five Predicted Potency Categories and Associated AI Limits for N-Nitrosamines

Potency Category	Recommended AI Limit (ng/day)	Comments
1	18	The recommended AI limit of 18 ng/day is equal to the class-specific TTC for *N*-nitrosamine impurities*. *N*-nitrosamines assigned to Category 1 are predicted to have high carcinogenic potency; however, the class-specific TTC for *N*-nitrosamine impurities is considered sufficiently protective to patients.
2	100	The recommended AI limit of 100 ng/day is representative of two potent, robustly tested *N*-nitrosamines, *N*-nitrosodimethylamine (NDMA) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-(butanone) (NNK), which have recommended AI limits of 96 ng/day and 100 ng/day, respectively. *N*-nitrosamines assigned to Category 2 are predicted to have carcinogenic potency no higher than NDMA and NNK.
3	400	Compared to Potency Category 2, *N*-nitrosamines in this category have lower carcinogenic potency due to, for example, the presence of a weakly deactivating structural feature. The recommended AI limit was set to reflect a 4-fold decrease in carcinogenic potency from Category 2.
4	1500	*N*-Nitrosamines assigned to Category 4 may be metabolically activated through an α-hydroxylation pathway but are predicted to be of low carcinogenic potency, for example, because the pathway is disfavored due to steric or electronic influences, or because clearance pathways are favored. The recommended AI limit of 1500 ng/day is set at the TTC per ICH M7**.
5	1500	*N*-Nitrosamines assigned to Category 5 are not predicted to be metabolically activated via an α-hydroxylation pathway due to steric hindrance or the absence of α-hydrogens, or are predicted to form unstable species that will not react with DNA. The recommended AI limit of 1500 ng/day is set at the TTC per ICH M7**.

* Assessment report Procedure under Article 5(3) of Regulation EC (No) 726/2004 Nitrosamine impurities in human medicinal products Procedure number: EMEA/H/A-5(3)/1490.

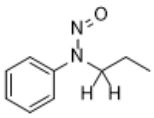
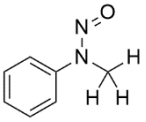
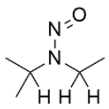
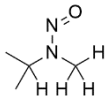
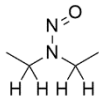
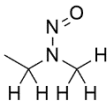
** See the International Council for Harmonisation guidance for industry *M7Assessment and Control of DNA Reactive (Mutagenic) Impurities in Pharmaceuticals To Limit Potential Carcinogenic Risk*. Threshold of Toxicological Concern (TTC) of 1.5 µg/day (1500 ng/day) as explained in ICH M7, represents an AI for any unstudied chemical that poses a negligible risk of carcinogenicity or other toxic effect.

Annex A. Calculation of potency score

For *N*-nitrosamines not assigned to Potency Category 5, the Potency Score is calculated as the sum of the α-Hydrogen Score (Table 2), Deactivating Feature Score (Table 3) and Activating Feature Score (Table 4) based on selected structural features present in the *N*-nitrosamine. The *N*-nitrosamine structure is expected to match exactly one of the α-hydrogen definitions in Table 2, but it may contain multiple or no structural features identified in Tables 3 and 4. In cases where one or more features from Tables 3 and 4 are contained in the *N*-nitrosamine, the Potency Score should be calculated as outlined in the box below. In cases where the *N*-nitrosamine contains no features from Tables 3 and 4, the Potency Score will be equal to the α-Hydrogen Score.

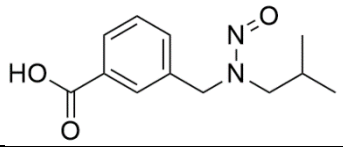
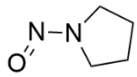
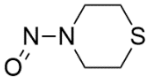
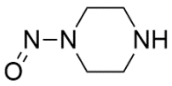
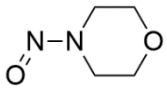
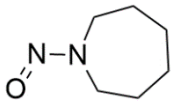
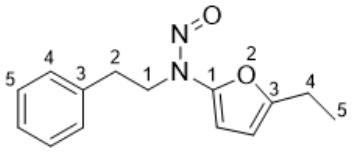
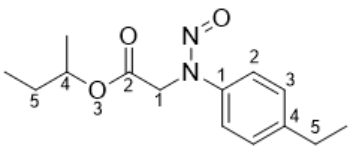
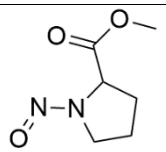
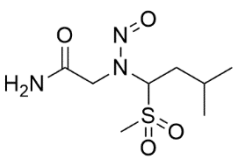
Potency Score = α**-Hydrogen Score** + **Deactivating Feature Score** (sum all scores for features present in the *N*-nitrosamine) + **Activating Feature Score** (sum all scores for features present in the *N*-nitrosamine)

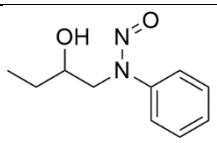
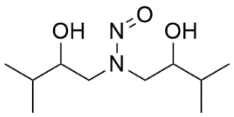
Table 2. Count of hydrogen atoms on each α-carbon (lowest count first) and corresponding α-Hydrogen Score. Examples are intended to be illustrative only and are not intended to be exhaustive.

Count of Hydrogen Atoms on Each α-Carbon, Lowest First	Example	α-Hydrogen Score
0,2		3*
0,3		2
1,2		3
1,3		3
2,2		1
2,3		1

***** A score of 3 applies when the methylene α-carbon is not part of an ethyl group. If the methylene α-carbon is part of an ethyl group, a score of 2 should be applied.

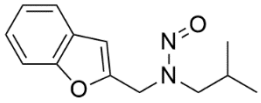
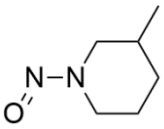
Table 3. List of deactivating features and associated scores. To calculate Deactivating Feature Score, sum the individual scores for all listed features present in the N-nitrosamine structure. Each deactivating feature row in the table may only be counted once. For N-nitrosamines where the N-nitroso group is within more than one ring, the feature score for only the smallest matching ring should be applied. Examples are intended to be illustrative only and are not intended to be exhaustive.

Deactivating Feature	Example	Individual Deactivating Feature Score
Carboxylic acid group anywhere on molecule		+3
N-nitroso group in a pyrrolidine ring		+3
N-nitroso group in a 6-membered ring containing at least one sulfur atom		+3
N-nitroso group in a 5- or 6-membered ring*		+2
N-nitroso group in a morpholine ring		+1
N-nitroso group in a 7-membered ring		+1
Chains of ≥5 consecutive non-hydrogen atoms (cyclic or acyclic) on both side of acyclic *N*-nitroso group. Not more than 4 atoms in each chain may be in the same ring.	 	+1
Electron-withdrawing group** bonded to α-carbon on only one side of *N*-nitroso group (cyclic or acyclic)		+1
Electron-withdrawing groups** bonded to α-carbons on both sides of *N*-nitroso group (cyclic or acyclic)		+2

Hydroxyl group bonded to β-carbon*** on only one side of *N*-nitroso group (cyclic or acyclic)		+1
Hydroxyl group bonded to β-carbon*** on both sides of *N*-nitroso group (cyclic or acyclic)		+2

*Excludes examples where *N*-nitroso group is in a pyrrolidine ring, a 6-membered ring containing at least one sulfur atom or a morpholine ring (all counted separately).
**Excludes carboxylic acid and aryl (counted separately), and ketone (conflicting data). Additional electron withdrawing group examples are limited to those described in Cross KP and Ponting DJ, 2021, Developing Structure-Activity Relationships for *N*-Nitrosamine Activity, Comput Toxicol, 20:100186, where they are referred to as "β-carbon electron withdrawing groups."
***β-Carbon must be in an sp^3 hybridisation state for this feature to apply.

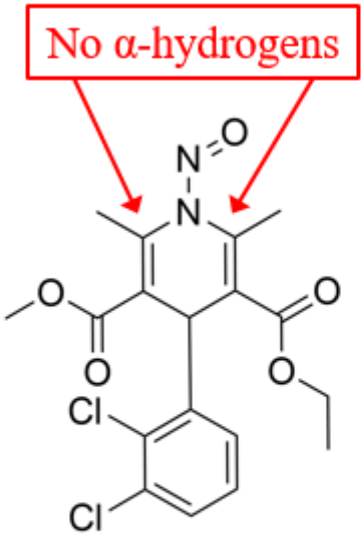
Table 4. List of activating features and associated scores. To calculate Activating Feature Score, sum the individual scores for all listed features present in the N-nitrosamine structure. Each activating feature row in the table may only be counted once. Examples are intended to be illustrative only and are not intended to be exhaustive.

Activating Feature	Example	Individual Activating Feature Score
Aryl group bonded to α-carbon (i.e., benzylic or pseudo-benzylic substituent on *N*-nitroso group)		-1
Methyl group bonded to β-carbon (cyclic or acyclic)		-1

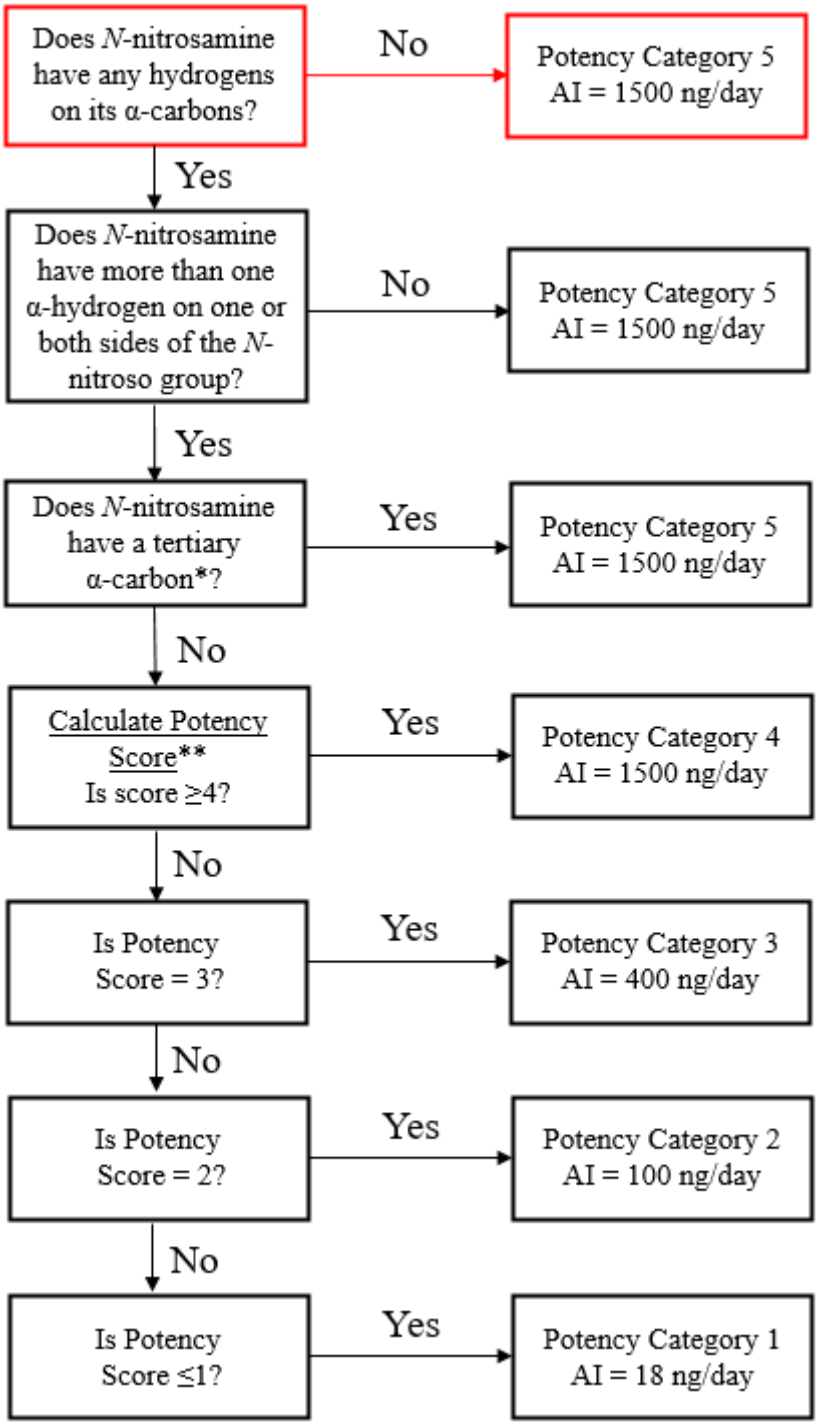
Annex B. Example of carcinogenic potency categorisation approach calculations based on flow chart

Example 1 – N-Nitroso-felodipine

Example 1 shows how the potency categorisation approach flow chart (Figure 2) can be applied to the *N*-nitrosamine, *N*-nitroso-felodipine. *N*-Nitroso-felodipine is placed in Potency Category 5 with an associated AI limit of 1500 ng/day.

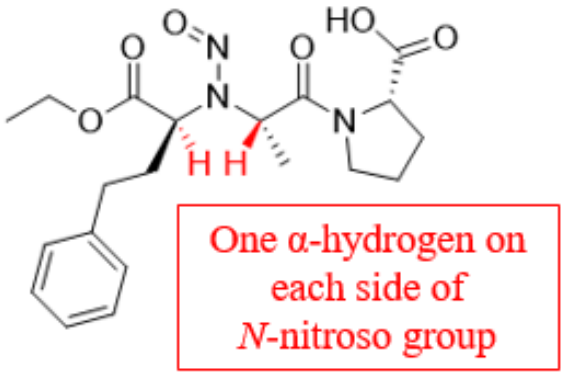


Potency Category 5	**AI = 1500 ng/day**

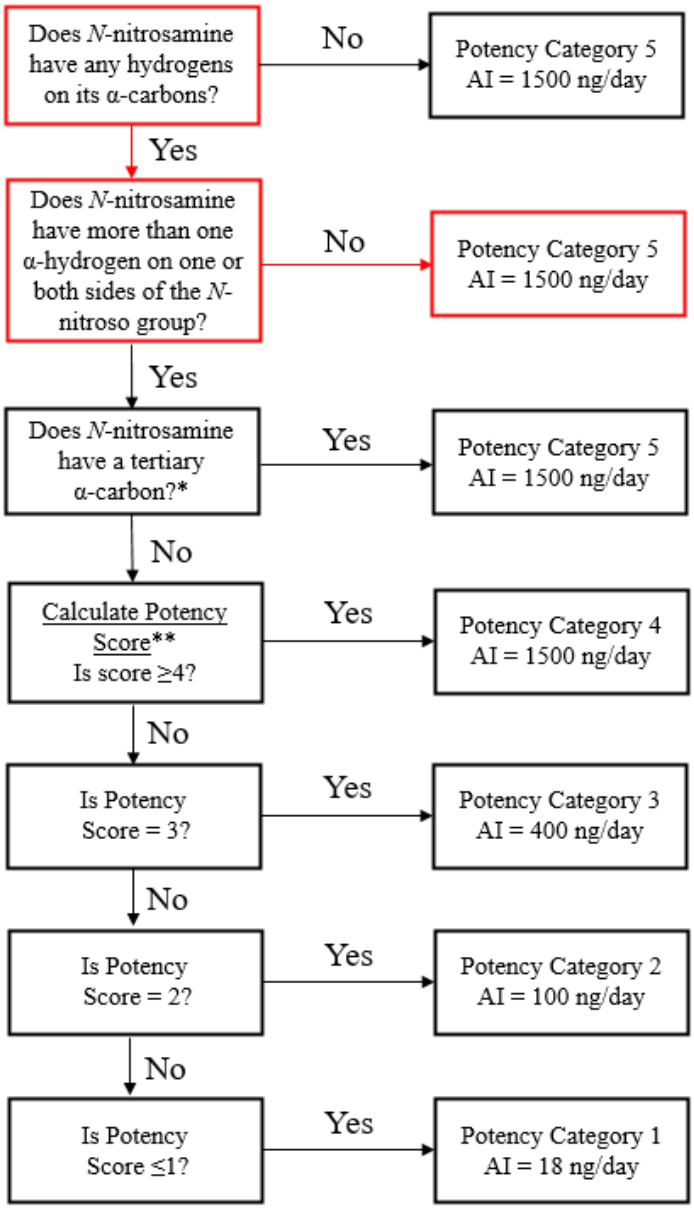

Does N-nitrosamine have any hydrogens on its α-carbons?
No
Potency Category 5 AI = 1500 ng/day
Yes
Does N-nitrosamine have more than one α-hydrogen on one or both sides of the N-nitroso group?
No
Potency Category 5 AI = 1500 ng/day
Yes
Does N-nitrosamine have a tertiary α-carbon*?
Yes
Potency Category 5 AI = 1500 ng/day
No
Calculate Potency Score**
Is score ≥4?
Yes
Potency Category 4 AI = 1500 ng/day
No
Is Potency Score = 3?
Yes
Potency Category 3 AI = 400 ng/day
No
Is Potency Score = 2?
Yes
Potency Category 2 AI = 100 ng/day
No
Is Potency Score ≤1?
Yes
Potency Category 1 AI = 18 ng/day

Example 2 – N-Nitroso-enalapril

Example 2 shows how the potency categorisation approach flow chart (Figure 2) can be applied to the *N*-nitrosamine, *N*-nitroso-enalapril. *N*-Nitroso-enalapril is placed in Potency Category 5 with an associated AI limit of 1500 ng/day.

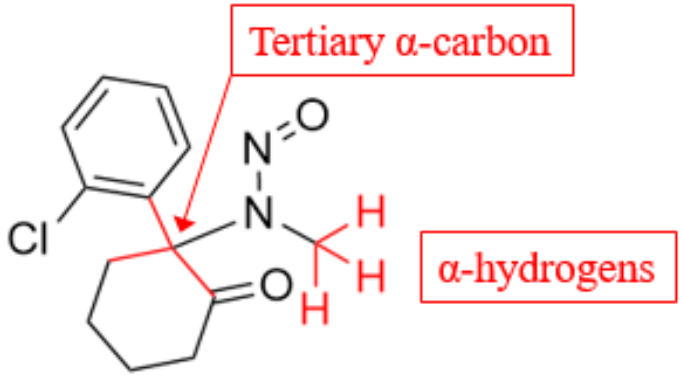


Potency Category 5	**AI = 1500 ng/day**

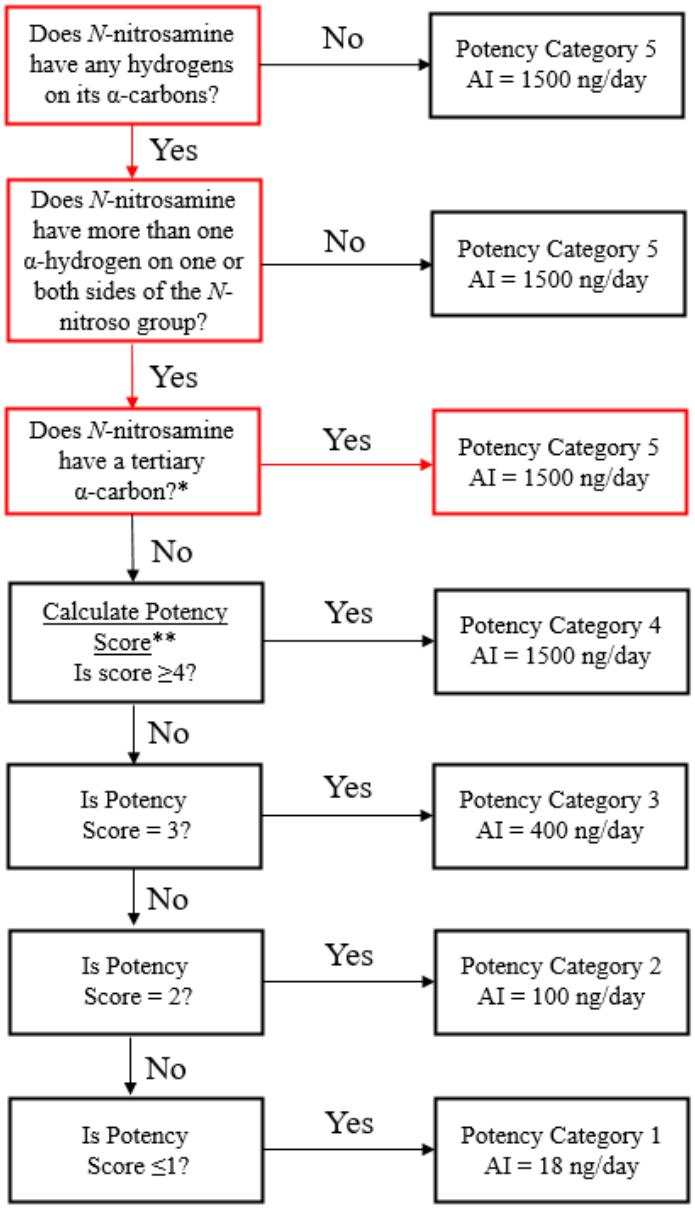

Does N-nitrosamine have any hydrogens on its α-carbons?
No
Potency Category 5 AI = 1500 ng/day
Yes
Does N-nitrosamine have more than one α-hydrogen on one or both sides of the N-nitroso group?
No
Potency Category 5 AI = 1500 ng/day
Yes
Does N-nitrosamine have a tertiary α-carbon?*
Yes
Potency Category 5 AI = 1500 ng/day
No
Calculate Potency Score** Is score ≥4?
Yes
Potency Category 4 AI = 1500 ng/day
No
Is Potency Score = 3?
Yes
Potency Category 3 AI = 400 ng/day
No
Is Potency Score = 2?
Yes
Potency Category 2 AI = 100 ng/day
No
Is Potency Score ≤1?
Yes
Potency Category 1 AI = 18 ng/day

Example 3 – N-Nitroso-ketamine

Example 3 shows how the potency categorisation approach flow chart (Figure 2) can be applied to the *N*-nitrosamine, *N*-nitroso-ketamine. *N*-Nitroso-ketamine is placed in Potency Category 5 with an associated AI limit of 1500 ng/day.

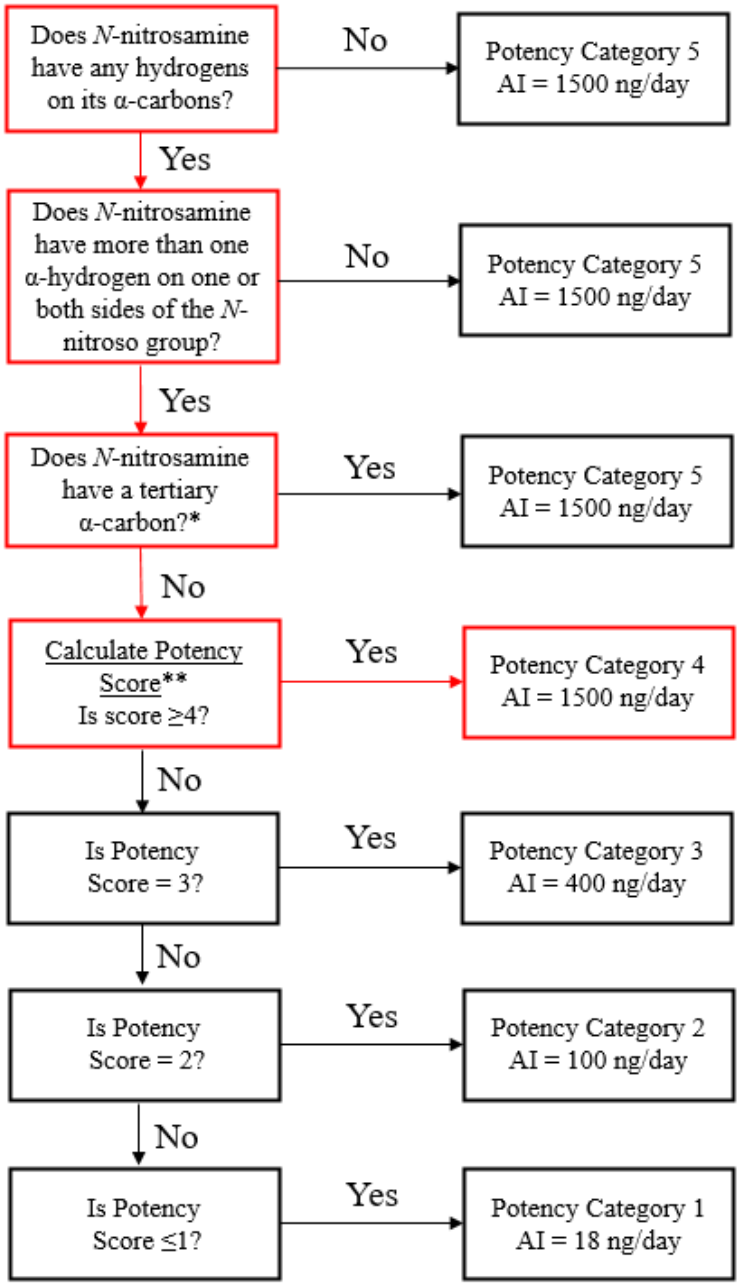


Potency Category 5	**AI = 1500 ng/day**


Does N-nitrosamine have any hydrogens on its α-carbons?
No
Potency Category 5 AI = 1500 ng/day
Yes
Does N-nitrosamine have more than one α-hydrogen on one or both sides of the N-nitroso group?
No
Potency Category 5 AI = 1500 ng/day
Yes
Does N-nitrosamine have a tertiary α-carbon?*
Yes
Potency Category 5 AI = 1500 ng/day
No
Calculate Potency Score** Is score ≥4?
Yes
Potency Category 4 AI = 1500 ng/day
No
Is Potency Score = 3?
Yes
Potency Category 3 AI = 400 ng/day
No
Is Potency Score = 2?
Yes
Potency Category 2 AI = 100 ng/day
No
Is Potency Score ≤1?
Yes
Potency Category 1 AI = 18 ng/day

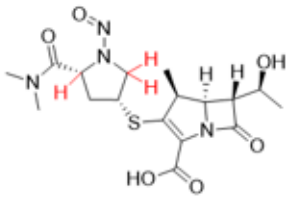
Example 4 – N-Nitroso-l-nebivolol

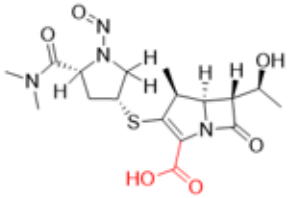
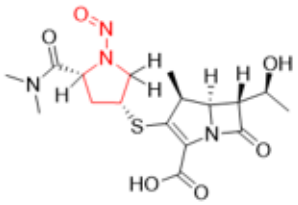
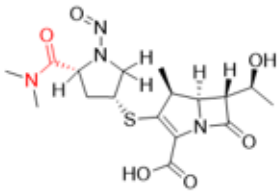
Example 4 shows how the potency categorisation approach flow chart (Figure 2) can be applied to the *N*-nitrosamine, *N*-nitroso-l-nebivolol. A Potency Score of 4 is calculated for *N*-nitroso-l-nebivolol, resulting in its placement in Potency Category 4 with an associated AI limit of 1500 ng/day.



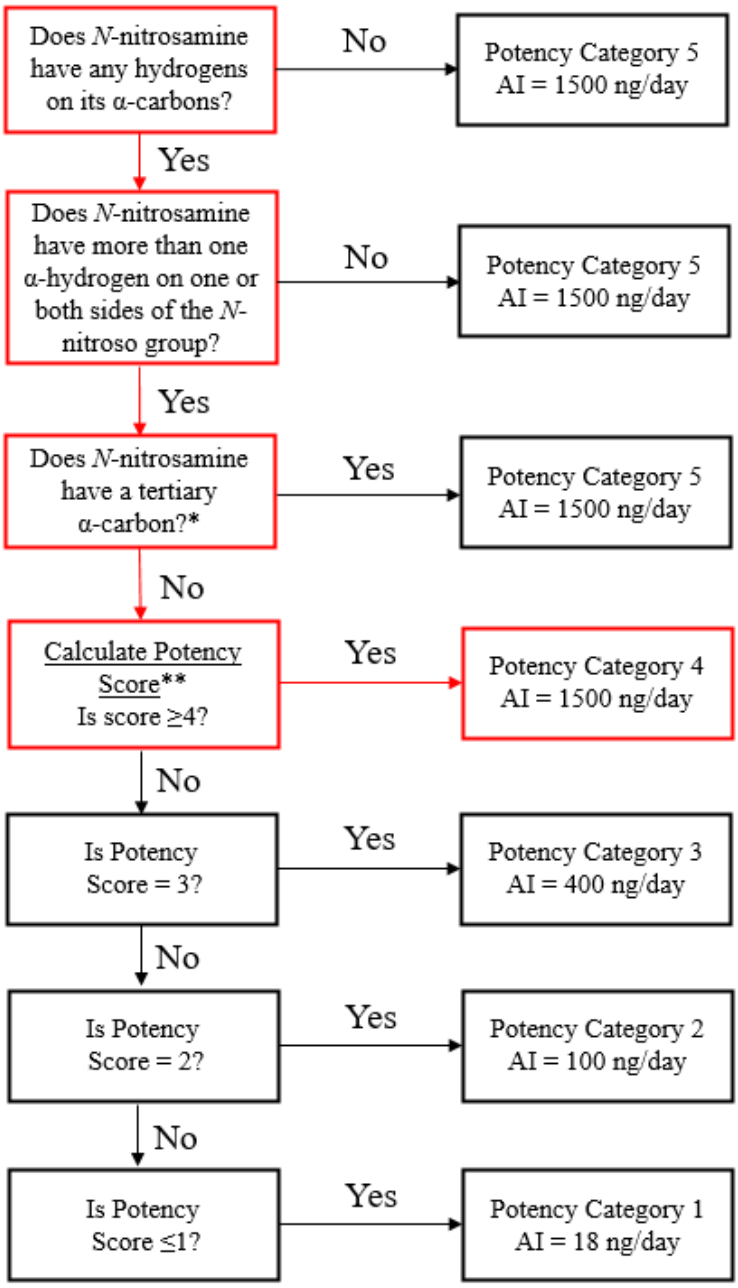
Example 5 – N-Nitroso-meropenem

Example 5 shows how the potency categorisation approach flow chart (Figure 2) can be applied to the *N*-nitrosamine, *N*-nitroso-meropenem. A Potency Score of 4 is calculated for *N*-nitroso-meropenem, resulting in its placement in Potency Category 4 with an associated AI limit of 1500 ng/day.

Count of α-Hydrogens	Score	Feature Highlighted in Red
1,2	3	

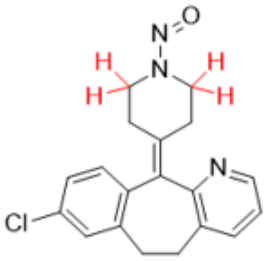
Deactivating Features	Score	Feature Highlighted in Red
Carboxylic acid group anywhere on molecule	+3	
N-nitroso group in a pyrrolidine ring	+3	
Electron-withdrawing group bonded to α-carbon on only one side of *N*-nitroso group (cyclic or acyclic)	+1	

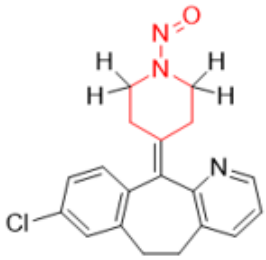
No Activating Features Present		
Potency Score = 3 + 3 + 3 + 1 = 10	**Potency Category 4**	**AI = 1500 ng/day**


Does N-nitrosamine have any hydrogens on its α-carbons?
No
Potency Category 5 AI = 1500 ng/day
Yes
Does N-nitrosamine have more than one α-hydrogen on one or both sides of the N-nitroso group?
No
Potency Category 5 AI = 1500 ng/day
Yes
Does N-nitrosamine have a tertiary α-carbon?*
Yes
Potency Category 5 AI = 1500 ng/day
No
Calculate Potency Score** Is score ≥4?
Yes
Potency Category 4 AI = 1500 ng/day
No
Is Potency Score = 3?
Yes
Potency Category 3 AI = 400 ng/day
No
Is Potency Score = 2?
Yes
Potency Category 2 AI = 100 ng/day
No
Is Potency Score ≤1?
Yes
Potency Category 1 AI = 18 ng/day

Example 6 – N-Nitroso-desloratadine

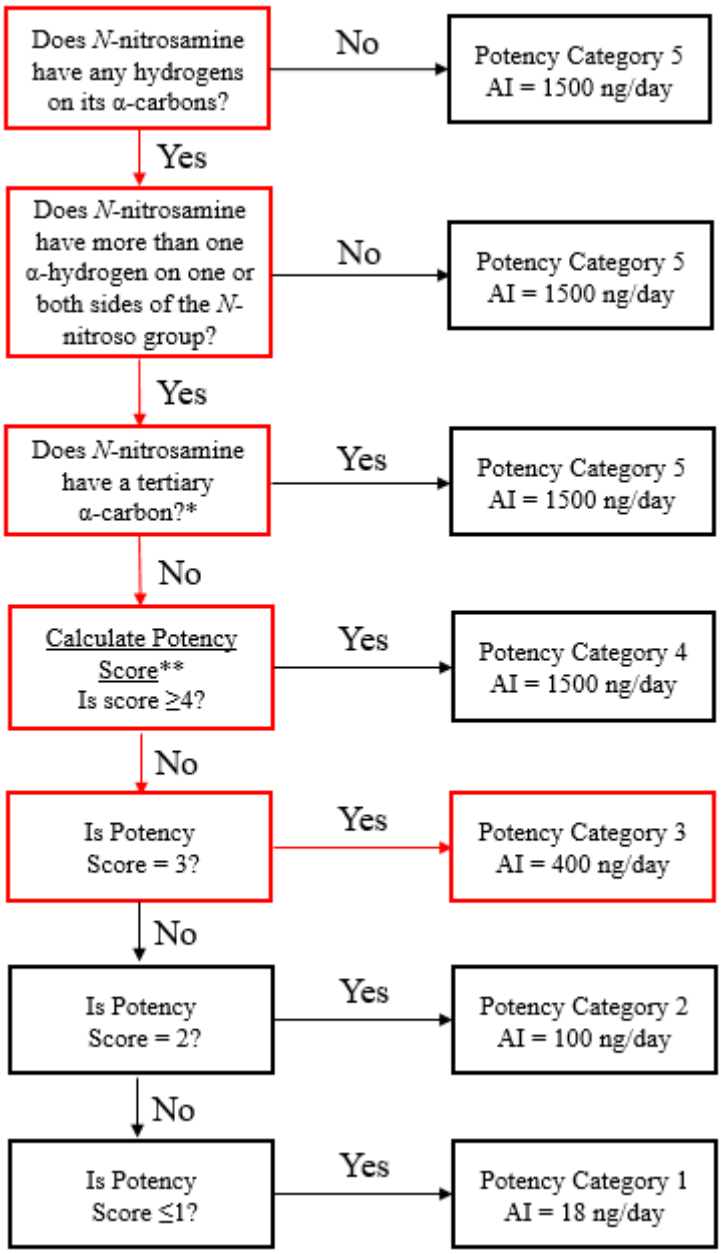
Example 6 shows how the potency categorisation approach flow chart (Figure 2) can be applied to the *N*-nitrosamine, *N*-nitroso-desloratadine. A Potency Score of 3 is calculated for *N*-nitroso-desloratadine, resulting in its placement in Potency Category 3 with an associated AI limit of 400 ng/day.

Count of α-Hydrogens	Score	Feature Highlighted in Red
2,2	1	

Deactivating Features	Score	Feature Highlighted in Red
N-nitroso group in a 5- or 6-membered ring	+2	

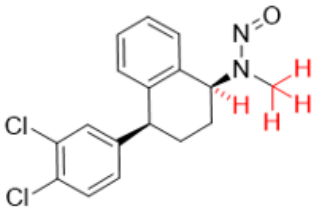
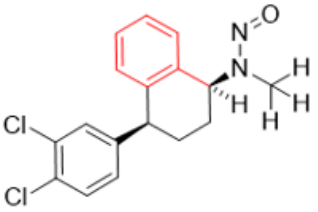
No Activating Features Present		

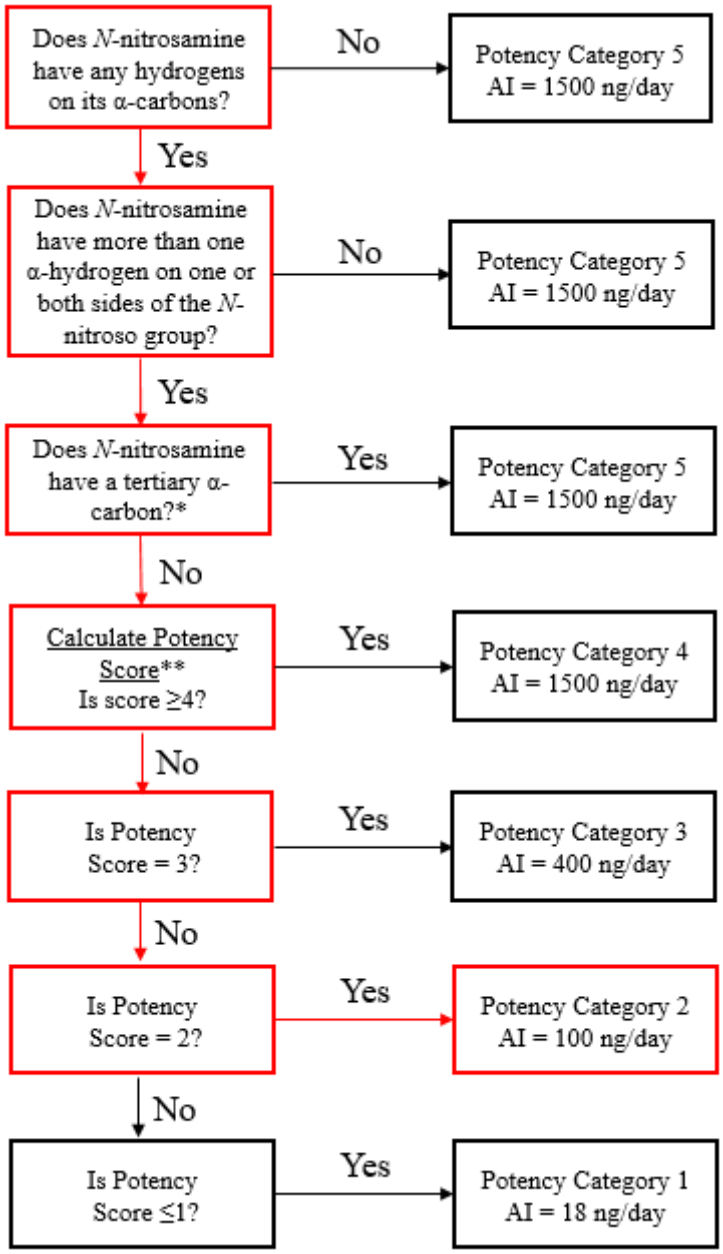
Potency Score = 1 + 2 = 3	Potency Category 3	AI = 400 ng/day


Does N-nitrosamine have any hydrogens on its α-carbons?
No
Potency Category 5 AI = 1500 ng/day
Yes
Does N-nitrosamine have more than one α-hydrogen on one or both sides of the N-nitroso group?
No
Potency Category 5 AI = 1500 ng/day
Yes
Does N-nitrosamine have a tertiary α-carbon?*
Yes
Potency Category 5 AI = 1500 ng/day
No
Calculate Potency Score**
Is score ≥4?
Yes
Potency Category 4 AI = 1500 ng/day
No
Is Potency Score = 3?
Yes
Potency Category 3 AI = 400 ng/day
No
Is Potency Score = 2?
Yes
Potency Category 2 AI = 100 ng/day
No
Is Potency Score ≤1?
Yes
Potency Category 1 AI = 18 ng/day

Example 7 – N-Nitroso-sertraline

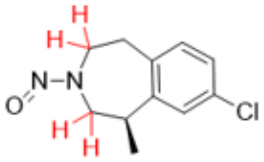
Example 7 shows how the potency categorisation approach flow chart (Figure 2) can be applied to the *N*-nitrosamine, *N*-nitroso-sertraline. A Potency Score of 2 is calculated for *N*-nitroso- sertraline, resulting in its placement in Potency Category 2 with an associated AI limit of 100 ng/day.

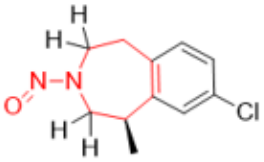
Count of α-Hydrogens	Score	Feature Highlighted in Red
1,3	3	
No Deactivating Features Present		
Activating Features	**Score**	**Feature Highlighted in Red**
Aryl group bonded to α-carbon (i.e., benzylic or pseudo-benzylic substituent on *N*-nitroso group)	-1	
Potency Score = 3 - 1 = 2	**Potency Category 2**	**AI = 100 ng/day**

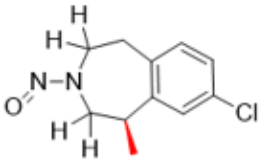

Does N-nitrosamine have any hydrogens on its α-carbons?
No
Potency Category 5 AI = 1500 ng/day
Yes
Does N-nitrosamine have more than one α-hydrogen on one or both sides of the N-nitroso group?
No
Potency Category 5 AI = 1500 ng/day
Yes
Does N-nitrosamine have a tertiary α-carbon?*
Yes
Potency Category 5 AI = 1500 ng/day
No
Calculate Potency Score** Is score ≥4?
Yes
Potency Category 4 AI = 1500 ng/day
No
Is Potency Score = 3?
Yes
Potency Category 3 AI = 400 ng/day
No
Is Potency Score = 2?
Yes
Potency Category 2 AI = 100 ng/day
No
Is Potency Score ≤1?
Yes
Potency Category 1 AI = 18 ng/day

Example 8 – N-Nitroso-lorcaserin

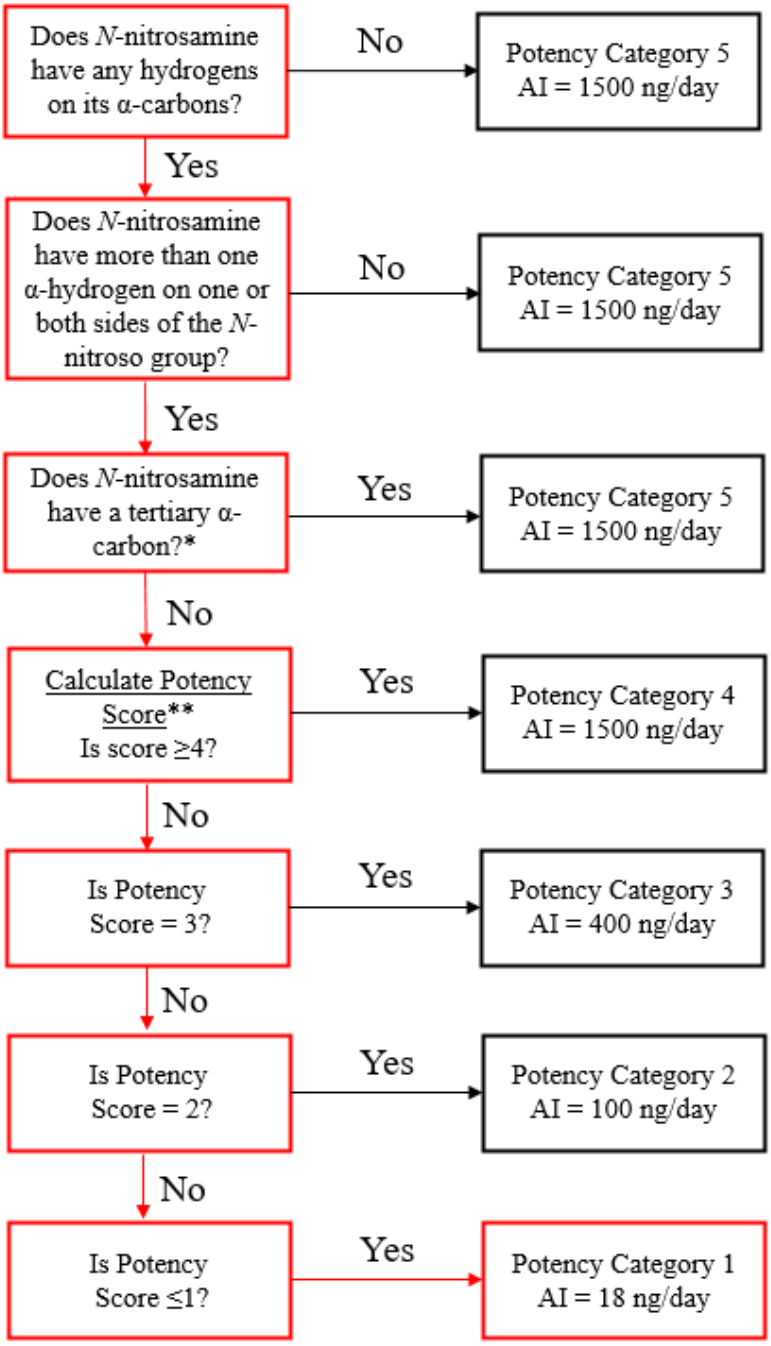
Example 8 shows how the potency categorisation approach flow chart (Figure 2) can be applied to the *N*-nitrosamine, *N*-nitroso-lorcaserin. A Potency Score of 1 is calculated for *N*-nitroso-lorcaserin, resulting in its placement in Potency Category 1 with an associated AI limit of 18 ng/day.

Count of α-Hydrogens	Score	Feature Highlighted in Red
2,2	1	

Deactivating Features	Score	Feature Highlighted in Red
N-nitroso group in a 7-membered ring	+1	

Activating Features	Score	Feature Highlighted in Red
Methyl group bonded to β-carbon (cyclic or acyclic)	-1	

Potency Score = 1 + 1 - 1 = 1	Potency Category 1	AI = 18 ng/day


Does N-nitrosamine have any hydrogens on its α-carbons?
No
Potency Category 5 AI = 1500 ng/day
Yes
Does N-nitrosamine have more than one α-hydrogen on one or both sides of the N-nitroso group?
No
Potency Category 5 AI = 1500 ng/day
Yes
Does N-nitrosamine have a tertiary α-carbon?*
Yes
Potency Category 5 AI = 1500 ng/day
No
Calculate Potency Score**
Is score ≥4?
Yes
Potency Category 4 AI = 1500 ng/day
No
Is Potency Score = 3?
Yes
Potency Category 3 AI = 400 ng/day
No
Is Potency Score = 2?
Yes
Potency Category 2 AI = 100 ng/day
No
Is Potency Score ≤1?
Yes
Potency Category 1 AI = 18 ng/day