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7-Azabicyclo[2.2.1]heptane as a structural motif to block mutagenicity of nitrosamines

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ABSTRACT

Nitrosamines are potent carcinogens and toxicants in the rat and potential genotoxins in humans. They are metabolically activated by hydroxylation at an α-carbon atom with respect to the nitrosoamino group, catalyzed by cytochrome P450. However, there has been little systematic investigation of the structure–mutagenic activity relationship of *N*-nitrosamines. Herein, we evaluated the mutagenicity of a series of 7-azabicyclo[2.2.1]heptane *N*-nitrosamines and related monocyclic nitrosamines by using the Ames assay. Our results show that the *N*-nitrosamine functionality embedded in the bicyclic 7-azabicylo[2.2.1]heptane structure lacks mutagenicity, that is, it is inert to α-hydroxylation, which is the trigger of mutagenic events. Further, the calculated α-C–H bond dissociation energies of the bicyclic nitrosamines are larger in magnitude than those of the corresponding monocyclic nitrosamines and *N*-nitrosodimethylamine by as much as 20–30 kcal/mol. These results are consistent with lower α-C–H bond reactivity of the bicyclic nitrosamines. Thus, the 7-azabicyclo[2.2.1]heptane structural motif may be useful for the design of nongenotoxic nitrosamine compounds with potential biological/medicinal applications.

1. Introduction

Nitrosamines, of which *N*-nitrosodimethylamine (NDMA) is a representative simple example, are well-known as potent carcinogens and toxicants in rats[1,2] and as potential genotoxins in humans.[3] Like many toxicants/mutagens, NDMA requires metabolic activation to manifest its toxicity, and hydroxylation at an α-carbon atom with respect to the nitrosoamino group, catalyzed by cytochrome P450 2E1, has been proposed to be the major metabolic activation pathway (Fig. 1).[4,5] The formation of α-hydroxy-NDMA (**B**, Fig. 1) is thought to involve intermediacy of an α-carbon radical or α-carbocationic species (**A**, Fig. 1), which is captured by a hydroxyl functionality.[6] The resultant α-hydroxy-NDMA is a kind of hemiaminal, which can undergo fragmentation reaction to form formaldehyde and an alkanediazohydroxide intermediate (**C**, Fig. 1), and this is transformed to methanediazonium ion (**D**, Fig. 1) upon elimination of water. The latter reactive species (**D**) can react with DNA to form alkylated adducts, such as O^6-methylguanine (O^6-MeG).[7,8] O^6-MeG is classed as a promutagenic lesion, and its formation has also been correlated with the cytotoxicity of methylating agents in rapidly dividing cells.[9] The metabolism of *N*-nitrosodialkylamines by rat liver microsomes has also been reported,[4,5] and short-lived radicals have been detected in the presence of rat liver microsomes.[10] On the other hand, there has been little systematic investigation of the structural requirements for mutagenic activity of *N*-nitrosamines. If we acquire this knowledge,[11,12] it may be possible to block the genotoxic activity of

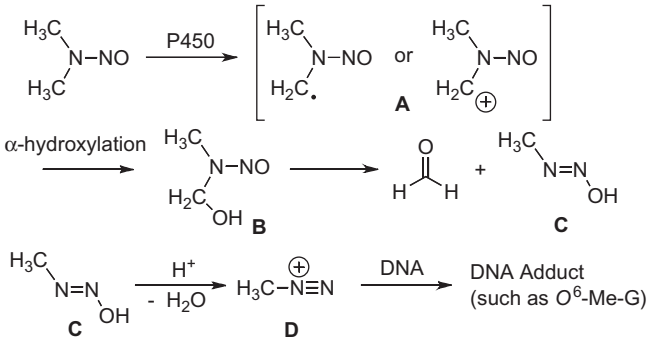

Figure 1. Metabolic oxidative activation of *N*-nitrosodimethylamine.

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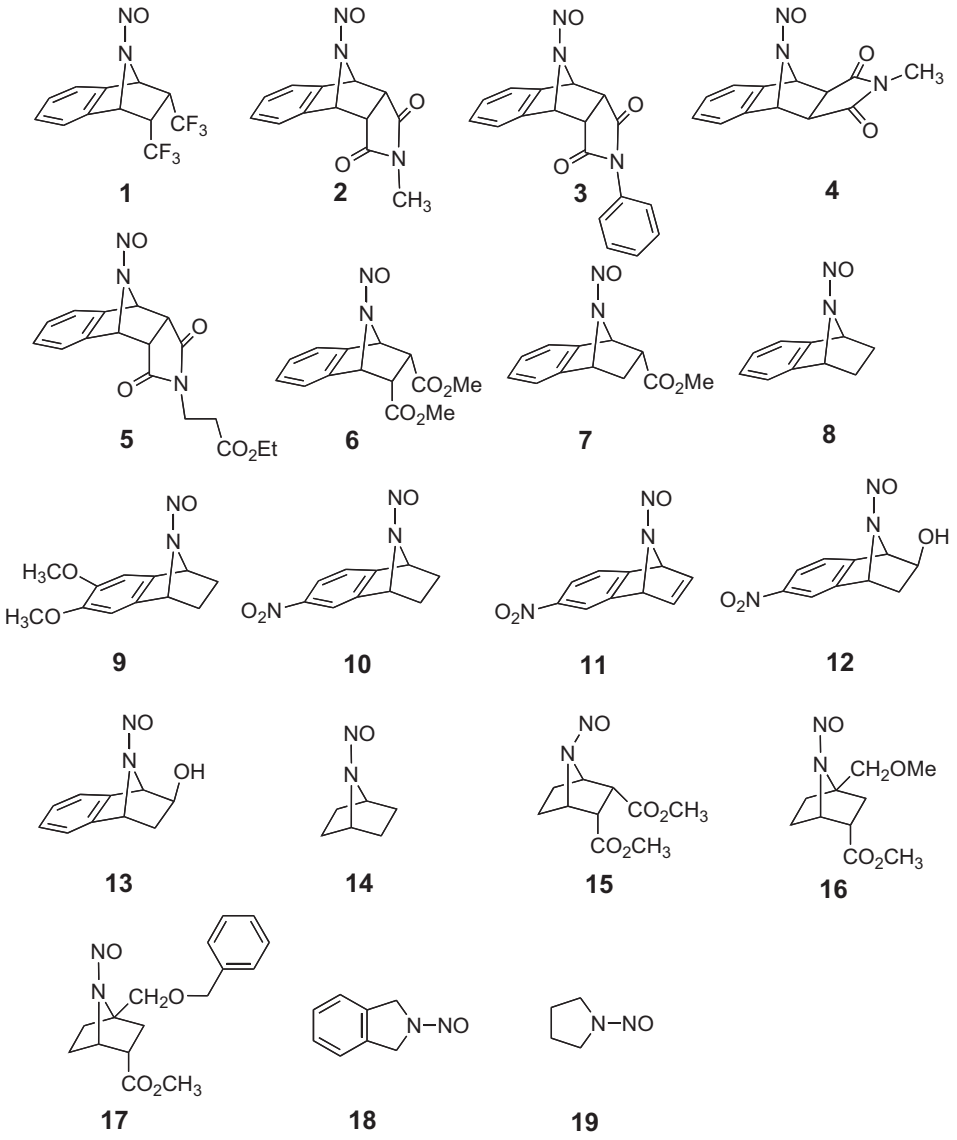


Scheme 1. Nitrosamines studied in this work.

nitrosamines, which would enable the nitrosamine functionality to be utilized in the molecular design of active pharmaceutical ingredients (API) in medicinal chemistry,[3] as well as bioactive molecules in biology.

7-Azabicyclo[2.2.1]heptane *N*-nitrosamines (**1–17**, Scheme 1) are *N*-nitroso derivatives of the bicyclic amine, 7-azabicyclo[2.2.1]-heptane. These nitrosamines constitute a new type of nitric oxide (NO) donors, which do not produce NO directly at neutral pH, but it can transnitrosate to thiols to generate *S*-nitrosothiols.[13] While these nitrosamines have potential applications in biochemistry and medicinal chemistry, via modulation of protein functions through *S*-nitrosation of cysteine residue(s),[14] there is great concern about their possible mutagenicity. Thus, we have evaluated the mutagenicity of these bicyclic nitrosamines, as well as related monocyclic nitrosamines, by using the Ames assay, which has been used widely for detecting DNA-damaging compounds.[15–17] In this assay, S9 mix, which consists of cytochrome P450s and an NADPH-regenerating system, is used for activating promutagens to active forms.

Herein, we determined the mutagenicity of 7-azabicyclo[2.2.1]-heptane *N*-nitrosamines and the corresponding monocyclic nitrosamines in the Ames assay, evaluated the structure–toxicity relationship, and calculated the α-C–H bond dissociation energies. Our results indicate that the 7-azabicyclo[2.2.1]heptane structural motif is effective to block the metabolic activation of nitrosamine to the proximate mutagenic species.

2. Results and discussion

2.1. Ames assay of bicyclic and monocyclic *N*-nitrosamines

2.1.1. Bicyclic nitrosamines

N-Nitrosamines of 7-azabicyclo[2.2.1]heptane were assayed for mutagenicity in the absence or presence of rat liver S9 mix in two strains, *Salmonella typhimurium* TA100 and TA98, which are used for detecting DNA-base substitution and frame-shift mutation, respectively.[15–17] Among 17 bicyclic nitrosamines (**1–17**, Scheme 1), 14 compounds were negative in the Ames assay (Figs. 2A–C), but three bearing aromatic dimethoxy groups (**9**) or an aromatic nitro group (**11** and **12**) showed apparent mutagenicity in the absence and presence of rat liver S9 mix in both TA100 and TA98 (Fig. 3A). In addition, nitrosamine (**3**) showed

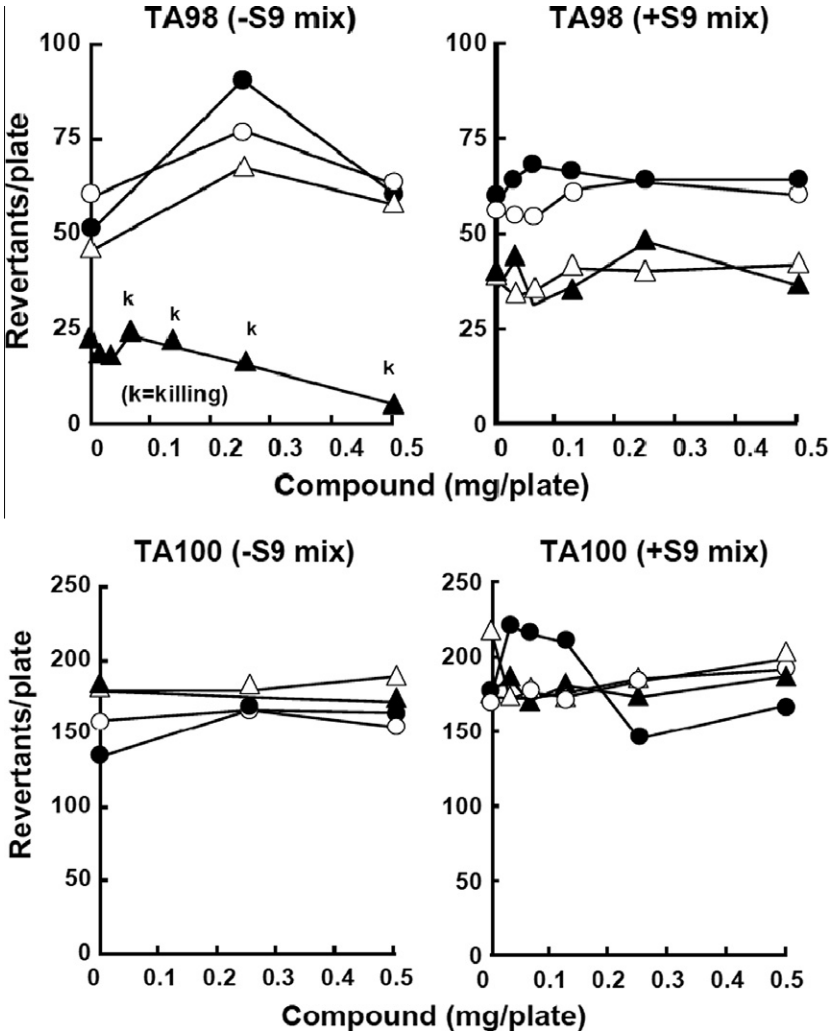


Figure 2A. Mutagenicity of compounds **1** (●), **2** (○), **3** (▲), and **4** (△) in *S. typhimurium* TA98 and TA100 in the presence of S9 mix or absence of S9 mix.

cytotoxicity in the absence of S9 mix in the case of the TA98 strain, while in the presence of S9 mix, no cytotoxicity or mutagenicity was detected in either strain.[18] This type of phenomenon, that is, a cytotoxicity-antagonizing effect of S9 mix has often been observed, and is considered to be due to the reaction of test compounds or their metabolites with proteins in S9 mix.[17]

2.1.2. Monocyclic nitrosamines

On the other hand, monocyclic nitrosamines (**18** and **19**) showed apparent mutagenicity only upon metabolic activation in the presence of rat liver S9 mix; in the absence of S9 mix, they were not mutagenic (Fig. 3B). Intriguingly, the mutagenic potencies of these monocyclic nitrosamines are different: the benzo derivative **18** showed strong activities in both TA100 and TA98, while the non-benzo derivative **19** showed significantly reduced activity in TA100 and little activity in TA98. The mutagenic activities of both **18** and **19** in TA98 were much weaker than those in TA100 (Fig. 3B). Thus, the mutagenicity of these nitrosamines arises from DNA-base substitution rather than DNA frame-shift mutation. This conclusion is consistent with a previous report.[19]

While the mutagenicity of the bicyclic nitrosamines **11** and **12** can be detected, the nitrosamine (**10**) bearing an aromatic nitro group showed no activity in the Ames assay with either TA98 or TA100. This is consistent with previous reports that nitrobenzene is negative in the Ames assay.[20–22] Also the mutagenicity of **11** and **12** can be detected regardless of metabolic activation, that is, in the absence or presence of S9 mix. The mutagenicity of both **11** and **12** in the absence of the S9 mix was stronger than that in the presence of the S9 mix. The olefin (**11**) and hydroxyl (**12**) functionalities are chemically interconvertible and may also be interconvertible in *Salmonella*.[23] Aromatic dimethoxy derivative **9** is also mutagenic, regardless of metabolic activation (Fig. 3A). The mutagenicity of **9** is also stronger in the absence of the S9 mix than that in the presence of the S9 mix in both TA100 and TA98.

These above results suggest that the *N*-nitrosamine functionality embedded in the bicyclic 7-azabicylo[2.2.1]heptane structure lacks mutagenicity, that is, the structure may be inert to metabolic α-hydroxylation, which is a trigger of mutagenic events in the case of generally carcinogenic *N*-nitrosodialkylamines. This is in strong contrast to the observed mutagenicity of the monocyclic nitrosamines (**18**, **19** and others), which are susceptible to enzymatic α-hydroxylation. Because three bicyclic *N*-nitrosamines (**9**, **11** and **12**) showed mutagenicity even in the absence of S9 mix, their mutagenicity is considered to be independent of the *N*-nitroso structure. At present the origin of their mutagenicity is unclear.

Therefore, it appears that the 7-azabicyclo[2.2.1]heptane structural motif can be useful for structural design of non-genotoxic nitrosamine compounds with potential for biological/medicinal applications.

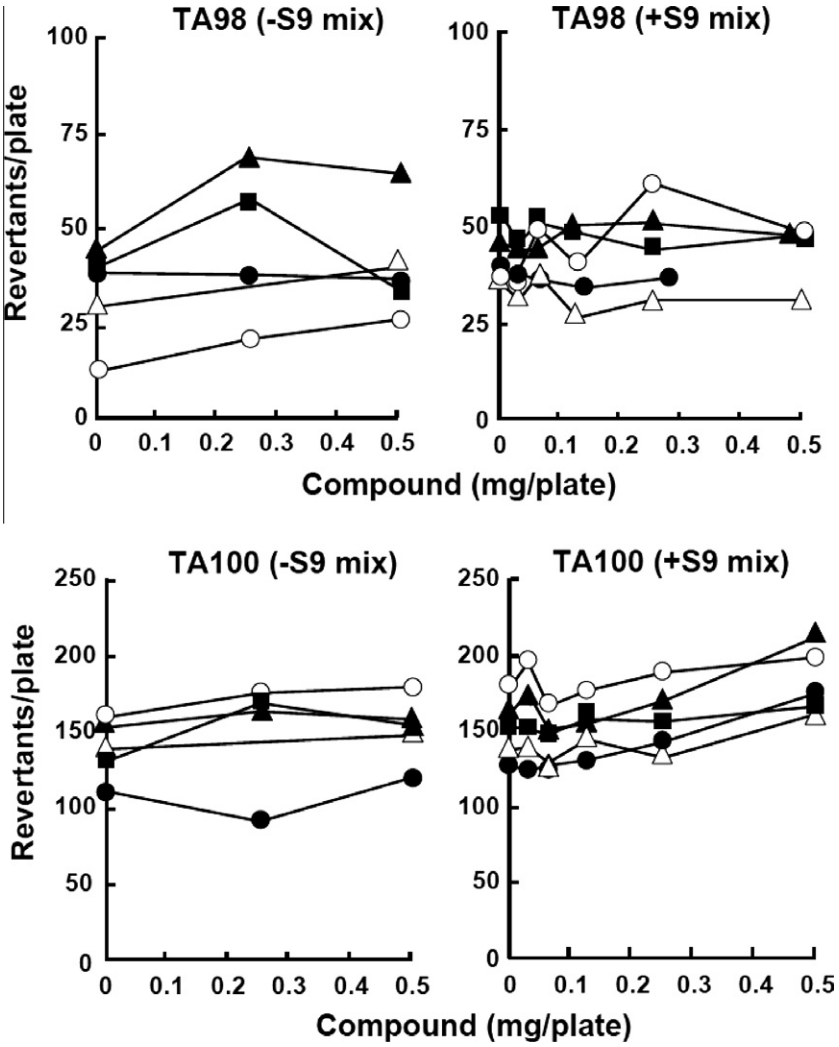


Figure 2B. Mutagenicity of compounds **5** (■), **6** (●), **7** (○), **8** (▲), and **10** (△) in *S. typhimurium* TA98 in the presence of S9 mix or absence of S9 mix.

2.2. Evaluation of ease of α-hydroxylation based on computed C–H bond dissociation energies

In order to rationalize the Ames assay results, we computationally evaluated the homolytic C–H bond dissociation energy (BDE) at the α-position of the nitrogen atom of the nitrosamine group. Previous studies have suggested the involvement of the carbon radical/carbocationic intermediate in the metabolic α-hydroxylation of *N*-nitrosamines (see Fig. 1).[1,2,7] Thus, the homolytic BDE of the relevant C–H bond can be used as a measure of the strength of the C–H bond and thus of the likelihood of homolytic bond cleavage, which leads to the putative α-carbon radical intermediates for α-hydroxylation (Fig. 4). The larger the magnitude of the BDE of the relevant C–H bond, the harder it is to form the carbon radical/carbocation intermediate,[24] and this corresponds to increasing resistance to metabolic α-hydroxylation, that is, inertness in the Ames assay.

BDE is experimentally defined as the enthalpy change at 298 K and 1 atm for the reaction A–B (g)→A·(g) + B·(g). The commonly used Hartree-Fock or perturbation (e.g., MP2, etc.) methods are not suitable for BDE calculations due to spin-contamination problems in dealing with open-shell systems.[25,26] More sophisticated ab initio methods such as QCISD and CCSD may perform better than HF and MP2 for the BDE calculations.[27] However, recent studies have shown that these more sophisticated methods may still have serious problems with some BDEs.[28] On the other hand, density functional theory (DFT) calculations usually do not show serious spin-contamination and, therefore, have been proposed to be reliable for open-shell systems.[29] At present, the most reliable way to calculate BDEs is to use composite ab initio methods, such as G3 and Complete Basis Set (CBS)-Q, which can provide predicted BDEs within 1–2 kcal/mol of the experimental values,[30–32] although these methods are very expensive in terms of CPU time and memory requirement. CBS-Q is a composite ab initio method, which starts with HF/6-31G(d) geometry optimization and frequency calculation, followed by MP2(FC)/6-31G(d) optimization. Then the single-point energy is calculated at MP2/6-311+G(3d2f,2df,2p), MP4(SDQ)/6-31+G(d(f),p), and QCISD(T)/6-31+G(d) levels. These energies are then extrapolated to the complete basis set limit.

Herein, we used the DFT methods to evaluate C–H BDE of these bicyclic and monocyclic nitrosamines (Table 1). The geometries of the nitrosamines and the corresponding carbon radical species were fully optimized at the Restricted (R) and Unrestricted (U) B3LYP/6-31G(d) level (method A), and the energies were estimated on the basis of the R(U)B3LYP/6-31G(d)-optimized structures by two DFT methods, R(U)B3LYP/6-311++G(d,p) (method B) and R(U)B3P86/6-311++G(d,p) (method C), at 273.15 K. These DFT methods were reported to show a good accuracy in calculation of

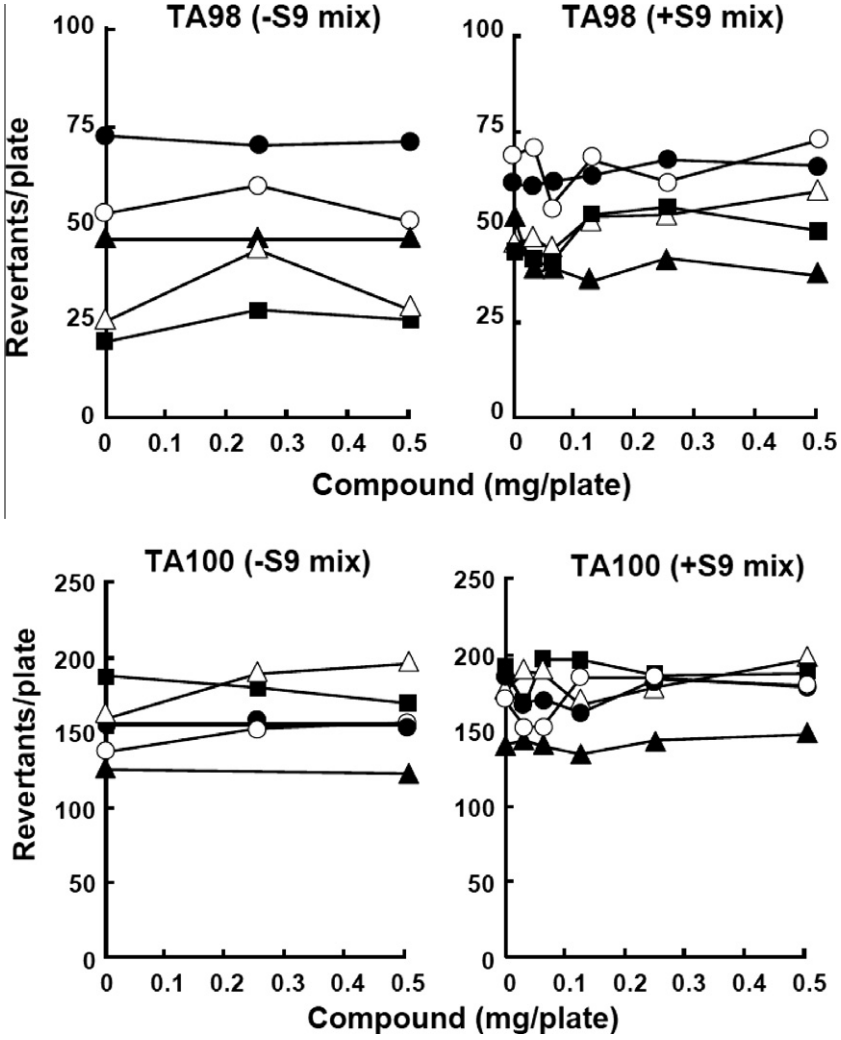


Figure 2C. Mutagenicity of compounds **13** (○), **14** (●), **15** (▲), **16** (△) and **17** (■) in *S. typhimurium* TA98 and TA100 in the presence of S9 mix or absence of S9 mix.

the C–H BDE, and in particular, the latter is comparable to the CBS-Q method.[29] Some selected simple molecules were also evaluated with the CBS-Q method (method D) at 293.15 K. Similar general trends in the order of magnitude of the BDEs of the relevant C–H bonds were obtained with both the DFT and CBS-Q calculation methods.

2.2.1. Bicyclic nitrosamines

In the case of the bicyclic nitrosamines, nitrogen pyramidalization and *cis*/*trans*-isomerism of the N–NO group take place.[33] Thus, four possible ground-state structures at most need to be considered in the cases of asymmetrical nitrosamines (see Fig. 9). Upon the putative homolytic cleavage of the C–H bond, there are two possible α-radical formations, that is, *anti*-radical and *syn*-radical with respect to the N═O group (Fig. 5, and see also Fig. 6). We compared the BDEs of these two unequivalent α-C–H bonds.

The C–H BDEs of two simple bicyclic nitrosamines, the benzo derivative **8** (Fig. 6) and the non-benzo derivative **14** (Fig. 7) were calculated. For the benzo derivative **8**, we calculated two ground-state conformers **8a** (*exo*-NO, bending of the nitrosamine group opposite to the aromatic ring) and **8b** (*endo*-NO, bending of the nitrosamine group toward the aromatic ring; **8a** is more stable than **8b**, though the energy difference is less than 1 kcal/mol), as well as four possible carbon radical species derived from **8a** and **8b** (Table 1 and Fig. 6). The BDEs of **8a** are 106.1 [108.9] kcal/mol and 106.7 [109.4] kcal/mol (method B and [method C]) for the formation of **8a**-*anti*-Rad and **8a**-*syn*-Rad, respectively. The BDEs of **8b** are 106.0 [108.6] kcal/mol and 106.4 [109.1] kcal/mol (method B and [method C]) for the formation of **8b**-*anti*-Rad and **8b**-*syn*-Rad, respectively (Table 1 and Fig. 6). The corresponding BDE values obtained by method D (CBS-Q) from **8b** are 119.3 kcal/mol (*anti*-radical) and 119.5 kcal/mol (*syn*-radical) at 298.15 K, respectively. Thus, the differences in BDEs between the two isomeric bonds, that is, *anti* and *syn* C–H bonds with respect to the nitrosoamino group, were small, and there is no significant effect of the initial ground-state conformation of the nitrosamine on the BDE values (Fig. 6). For the non-benzo derivative **14**, the BDE values were in a similar range of magnitude to those of the benzo-derivative **8**: for the formation of **14**-*anti*-Rad and **14**-*syn*-Rad, the BDEs are 105.3 [107.9] kcal/mol and 105.7 [108.3] kcal/mol (method B and method C), respectively (Table 1). The corresponding value obtained by method D for the least-energy process to generate **14**-*anti*-Rad is 110.6 kcal/mol at 298.15 K. These BDE values of **8** and **14** are comparable to or rather larger than those of the C–H bond of simple alkanes, such as cyclopentane (93.1 kcal/mol (method C) and 96.5 kcal/mol (method D)), respectively.[29] This is consistent with the notion that the resultant bridgehead carbon radicals (**8**-Rad and **14**-Rad, Fig. 4) are not

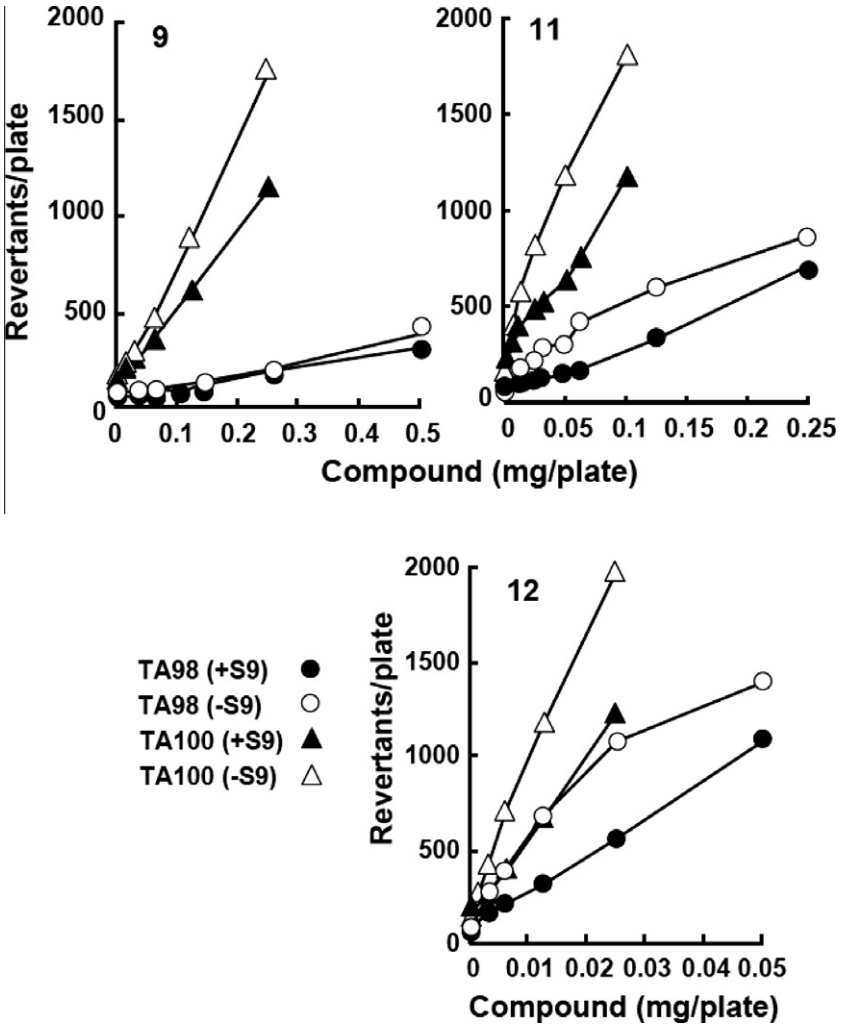


Figure 3A. Mutagenicity of compounds **9**, **11** and **12** in *S. typhimurium* strains in the presence or absence of S9 mix.

subject to conjugative stabilization by the adjacent nitrogen atom of the N–(NO) group and the fused benzene ring, probably because of the structural features of the present bicyclic system (see Fig. 8).

2.2.2. Monocyclic nitrosamines

In the series of monocyclic nitrosamines, optimized structures showed that the nitrogen atom of the N–(NO) group of benzo **18** is planar, while non-benzo **19** has a slightly pyramidalized nitrogen atom (see Fig. 7 and see Section 2.3). The BDEs of the benzo derivative **18** are 74.2 [76.9] and 74.6 [77.4] kcal/mol (method B and [method C]) for generation of **18**-*anti*-Rad and **18**-*syn*-Rad, respectively. The corresponding value obtained by method D (CBS-Q) for the least-energy process to generate **18**-*anti*-Rad is 78.7 kcal/mol at 298.15 K (Table 1). For the non-benzo derivative **19**, the BDEs are 81.7 [85.9] and 83.4 [84.7] kcal/mol for generation of **19**-*anti*-Rad and **19**-*syn*-Rad (method B and [method C]), respectively. The corresponding value obtained by method D (CBS-Q) for generation of **19**-*anti*-Rad is 89.0 kcal/mol at 298.15 K. This value is comparable in magnitude to that of NDMA (89.2 kcal/mol, method D) for the generation of NDMA-*syn*-Rad (Table 1). It was evident that the BDE values of the monocyclic nitrosamines (**18** and **19**) and NDMA are smaller in magnitude than those of the corresponding bicyclic nitrosamines (**8** and **14**) by about 20–30 kcal/mol. These results are consistent with the high reactivity of the α-C–H bond of the monocyclic nitrosamines and NDMA, which show positive Ames assay responses. It is noteworthy that the BDE of the benzo derivative **18** is smaller than that of the non-benzo derivative **19** by 10 kcal/mol. This indicates that the benzene ring stabilizes the resultant carbon radical in the case of **18** (see Fig. 8), which is consistent with the strongly positive Ames assay result of **18** as compared with the weakly positive response of **19** (Fig. 3B).

2.3. Structural difference of carbon radicals derived from bicyclic and monocyclic nitrosamines

2.3.1. Monocyclic nitrosamines

Nitrosamine **19** has sp^3-hybridized carbons (CH_2) at the α-position of the nitrosamino group. The nitrosamine nitrogen atom of **19** is only very slightly pyramidalized: θ_1 (the summation of the three bond angles of the nitrogen atom) is 359.2° (360.0° when the nitrogen atom is planar) (Fig. 7). Upon radical formation, the monocyclic radical **19**-Rad takes a planar structure with respect to both the radical carbon center and the nitrosoamino nitrogen atom: **19**-*anti*-Rad: θ_1: 360.0°; θ_2 (the summation of the three bond angles of the radical carbon atom) is 359.8°; **19**-*syn*-Rad: θ_1: 360.0°; θ_2: 360.0°. The N–C(radical) bond length is also shortened as compared with the corresponding N–C bond of the starting nitrosamine **19**: **19**-*anti*-Rad: d_1: 1.349 Å versus 1.465 Å

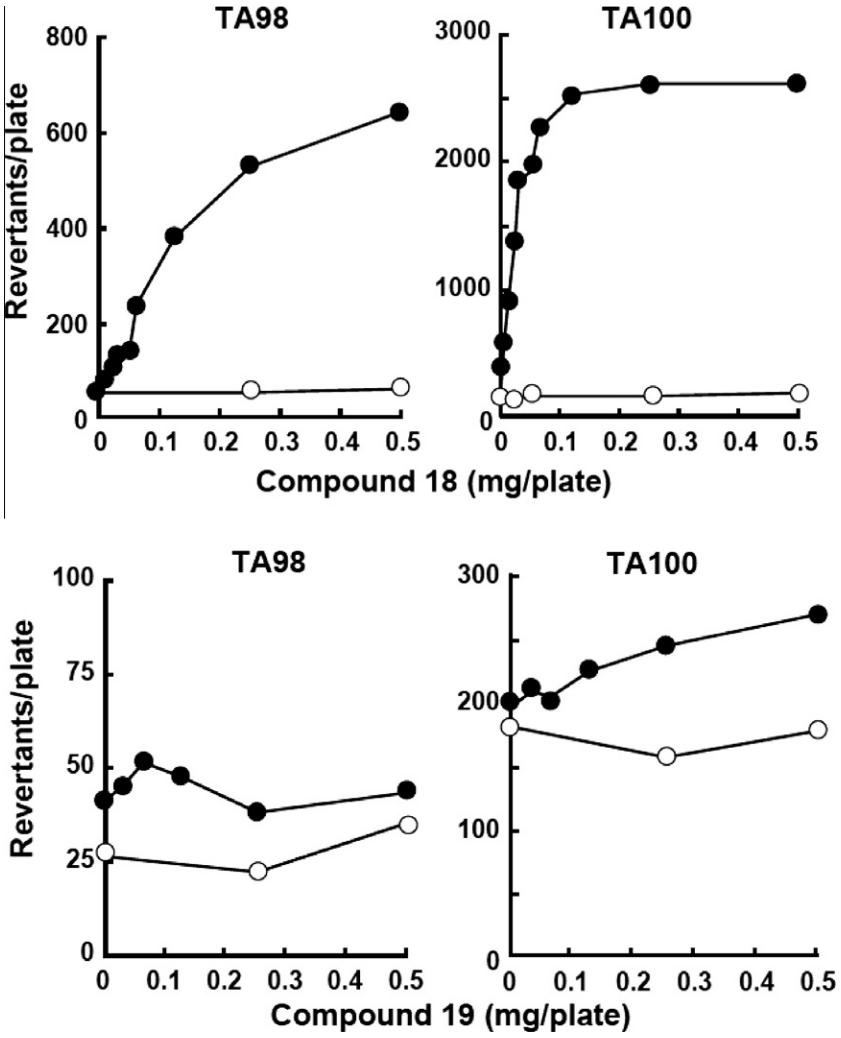


Figure 3B. Mutagenicity of compounds **18** and **19** in *S. typhimurium* strains in the presence (●) or absence of S9 mix (○).

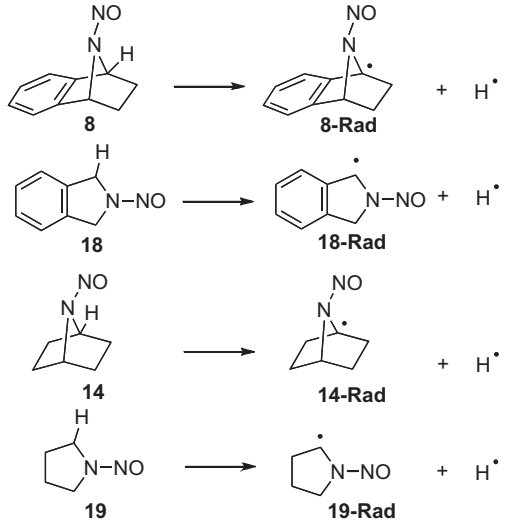


Figure 4. Bond dissociation energy of relevant C–H bonds.

(**19**); **19**-*syn*-Rad: d_2: 1.339 Å versus 1.469 Å (**19**) (Fig. 7). These structural changes upon carbon radical formation are consistent with a large contribution of the resonance structure, **19**-Rad-II (Fig. 8), that is, an increase of N–C double bond character.

2.3.2. Bicyclic nitrosamines

On the other hand, the geometrical changes upon formation of the corresponding carbon radical were significantly attenuated in the cases of the bicyclic nitrosamines (Fig. 7). The nitrosoamino nitrogen atom was pyramidalized (i.e., the summation of the three bond angles of the nitrogen atom is less than 360°) in the starting bicyclic nitrosamine **14**: θ_1 is 339.9°. Upon radical formation, the bridgehead radicals (**14**-*anti*-Rad and **14**-*syn*-Rad) maintain non-planar structures with respect to the nitrosoamino nitrogen atom and also the carbon radical center: **14**-*anti*-Rad: θ_1: 341.3°, θ_2: 320.4°; **14**-*syn*-Rad: θ_1: 340.0°; θ_2: 319.4°. The shortening of the N–C(radical) bond length as compared with the corresponding N–C bond of the starting nitrosamine **14** is rather small: **14**-*anti*-Rad: d_1: 1.458 Å versus 1.483 Å (**14**); **14**-*syn*-Rad: d_2: 1.467 Å versus 1.475 Å (**14**). These structural features are consistent with the postulate that the contribution of the resonance structure **14**-Rad-II (Fig. 8) is small and the formation of the carbon radical at the bridgehead position is difficult.

2.4. C–H Bond dissociation energies of the mutagenic bicyclic nitrosamines

We also calculated the BDEs of the α-C–H bonds of the mutagenic bicyclic nitrosamines (**9**, **11** and **12**) for comparison with those of the inert bicyclic nitrosamines (e.g., **10**) (Table 1). In the

Table 1
Calculated bond dissociation energies (kcal/mol)

Carbon radical species[e]	Geometry of NO[f]	Method A R(U)B3LYP[a,b] 6-31G(d)	Method B R(U)B3LYP[b,c] 6-311G++(d,p)	Method C R(U)B3P86[b,c] 6-311++G(d,p)	Method D CBS-Q[d]
Bicyclic nitrosamines					
8a-*anti*-Rad	*exo*	106.2	106.1	108.9	—[g]
8a-*syn*-Rad	*exo*	106.8	106.7	109.4	—[g]
8b-*anti*-Rad	*endo*	106.1	106.0	108.6	119.3
8b-*syn*-Rad	*endo*	106.6	106.4	109.1	119.5
9-*anti*-Rad	*exo-cis*	105.7	105.4	108.1	—[h]
9-*syn*-Rad	*exo-cis*	106.3	106.2	108.8	—[h]
10-*anti*-Rad	*exo-cis*	106.8	106.7	109.3	—[h]
10-*syn*-Rad	*exo-cis*	107.6	107.1	109.7	—[h]
11-*anti*-Rad	*exo-trans*	107.6	107.7	110.3	—[h]
11-*syn*-Rad	*exo-trans*	108.0	108.1	110.7	—[h]
12-*anti*-Rad	*exo-trans*	106.9	106.9	109.6	—[h]
12-*syn*-Rad	*exo-trans*	106.8	107.2	109.8	—[h]
14-*anti*-Rad	—	105.6	105.3	107.9	110.6
14-*syn*-Rad	—	105.9	105.7	108.3	—[h]
Monocyclic nitrosamines and NDMA					
18-*anti*-Rad	—	74.6	74.2	76.9	78.7
18-*syn*-Rad	—	75.4	74.6	77.4	—[h]
19-*anti*-Rad	—	83.1	81.7	85.9	89.0
19-*syn*-Rad	—	83.7	83.4	84.7	—[h]
NDMA-*anti*-Rad	—	87.9	86.2	89.3	91.3
NDMA-*syn*-Rad	—	86.1	84.3	87.4	89.2

[a] Geometries were fully optimized.
[b] Zero-point energies (obtained by B3LYP/6-31G(d)) were corrected.
[c] Structures optimized on B3LYP/6-31G(d) were used.
[d] At 298.15 K.
[e] *Anti* and *syn*-radicals (Rad) are α-carbon radicals with the radical center *anti* and *syn* with respect to the (N)–NO group, respectively. See Figure 5.
[f] *Exo*-NO and *endo*-NO refer to the bending of the nitrosoamine group with respect to the aromatic ring; *cis*-NO and *trans*-NO refer to the orientation of (N)–NO group with respect to the aromatic substituent. See Figure 9.
[g] The calculations were not convergent.
[h] Not determined.

cases of the asymmetrical nitrosamines **11** and **12**, there are four possible ground-state conformers (**a**: *exo-cis*-NO, **b**: *exo-trans*-NO, **c**: *endo-cis*-NO, **d**: *endo-trans*-NO; *exo*-NO and *endo*-NO define the direction of bending of the nitrosoamine group, see also Figure 6; *cis*-NO and *trans*-NO refer to the orientation of the (N)–NO group with respect to the aromatic substituent) (for **11a–d**, Fig. 9). The relative energy differences among the four conformers were small, that is, within 0.3–0.7 kcal/mol. The magnitudes of the C–H BDE were rather constant, regardless of the initial conformation, in the case of **8** (Table 1 and Fig. 6). Thus, we calculated the C–H BDE values of **9**–**12** on the basis of the least-energy ground-state conformers (Table 1). The BDEs (obtained by method C) of mutagenic **9** (conformer **9a** (*exo-cis*-NO): 108.1 and 108.8 kcal/mol), **11** (conformer **11b** (*exo-trans*-NO): 110.3 and 110.7 kcal/mol) and **12** (conformer **12b** (*exo-trans*-NO): 109.6 and 109.8 kcal/mol) for the *anti*-C–H bond and the *syn*-C–H bonds are similar in magnitude to those of Ames-inert **10** (conformer **10a** (*exo-cis*-NO): 109.3 and 109.7 kcal/mol), respectively. These similar magnitudes of the BDEs are consistent with the idea that all these bicyclic nitrosamines have similar chemical inertness to α-C–H bond cleavage. That is, the observed mutagenicity of the bicyclic nitrosamines (**9**, **11** and **12**) is unrelated to α-hydroxylation.

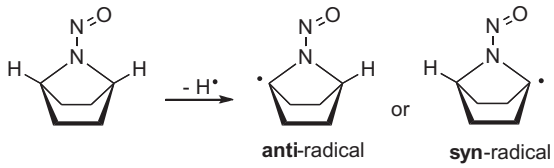


Figure 5. Two isomeric C–H bonds at the α-position of nitrosamine.

3. Conclusion

While nitrosamines are generally regarded as carcinogenic, the *N*-nitrosamines of 7-azabicyclo[2.2.1]heptane are essentially negative in the Ames assay with the TA100 and TA98 strains. Some bicyclic nitrosamines show a positive Ames assay response without enzymatic activation, implying that the activity is independent of the nitrosamine functionality. The calculated BDEs of the α-C–H bonds of the present bicyclic nitrosamines (**8–12**, and **14**) are larger in magnitude than those of the corresponding monocyclic nitrosamines (**18** and **19**) and NDMA, and the difference is as large as 20–30 kcal/mol. These results are consistent with low α-C–H bond reactivity of the present bicyclic nitrosamines, and consequently with low mutagenicity in the Ames assay, whereas the monocyclic nitrosamines and NDMA have higher α-C–H bond reactivity, which is consistent with positive Ames assay responses. These findings indicate that 7-azabicyclo[2.2.1]heptane is available as a structural motif for the design of non-genotoxic nitrosamine compounds.

4. Experimental section

4.1. Materials, and reagents for biological assay

Sodium ammonium hydrogen phosphate tetrahydrate was purchased from Merck (Darmstadt, Germany). Bacto agar and bacto nutrient broth were obtained from Becton Dickinson Microbiology Systems (Sparks, USA). Other reagents used were purchased from Wako Pure Chemical Industries (Osaka, Japan). S9/cofactor A set for the Ames test was purchased from Oriental Yeast Co., Ltd (Tokyo, Japan). Dr. T. Nohmi (National Institute of Health Sciences, Tokyo, Japan) kindly provided *S. typhimurium* TA100 and TA98.

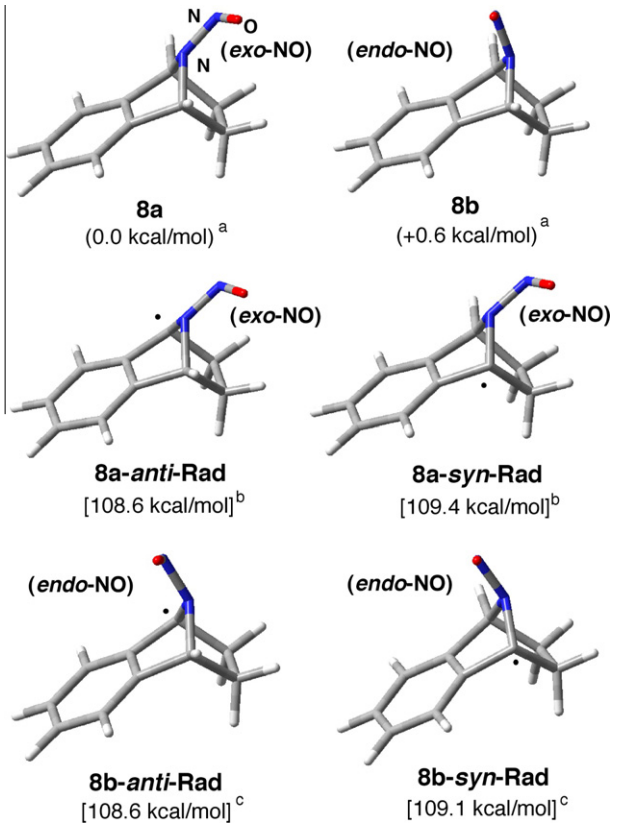


Figure 6. Ground-state conformers (**8a** and **8b**) and four possible carbon radical species of benzonitrosamine 8. Two conformers, **8a** (*exo*-NO) and **8b** (*endo*-NO) were calculated. *Exo*-NO and *endo*-NO refer to the bending of the nitrosamine group with respect to the aromatic ring. The energy difference obtained by method C is shown in parentheses. (b) The C—H BDEs from conformer **8a** (method C) are shown in brackets. (c) The C—H BDEs from conformer **8b** (method C) are shown in brackets.

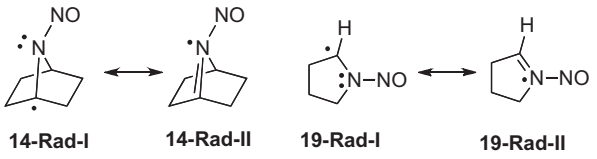


Figure 8. Resonance structures to stabilize radicals.

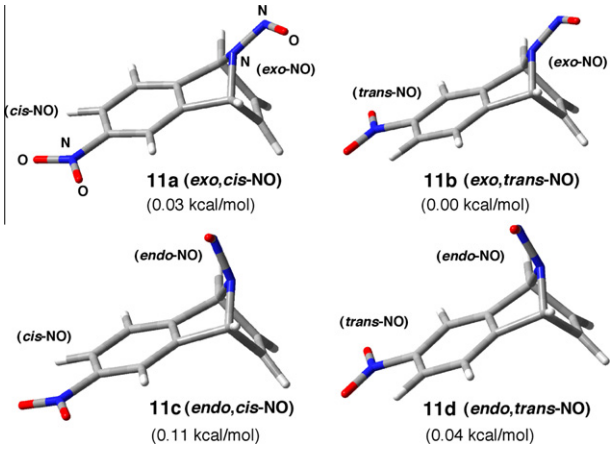


Figure 9. Four possible ground-state conformers of **11a**–**d**. Relative energies (by method C) are shown in parentheses. *Exo*-NO and *endo*-NO refer to the bending of the nitrosoamine group with respect to the aromatic ring (see also Fig. 6); *Cis*-NO and *trans*-NO refer to the orientation of (N)–NO group with respect to the aromatic substituent.

Several tester strains have been employed for assays of mutagenesis involving *N*-nitrosamines in the presence of rat liver S9 mix. The *his* G46 *Salmonella* tester strains TA1535 and TA100 are sensitive mainly to damage through base substitution mutation.[34] Furthermore some *N*-nitrosamines having a bulky group in the structure, for example, *N*-nitrosophenylurea[35] and *N*-nitrosomethylbenzylamine,[36] are weak frame-shift mutagens in *S. typhimurium*

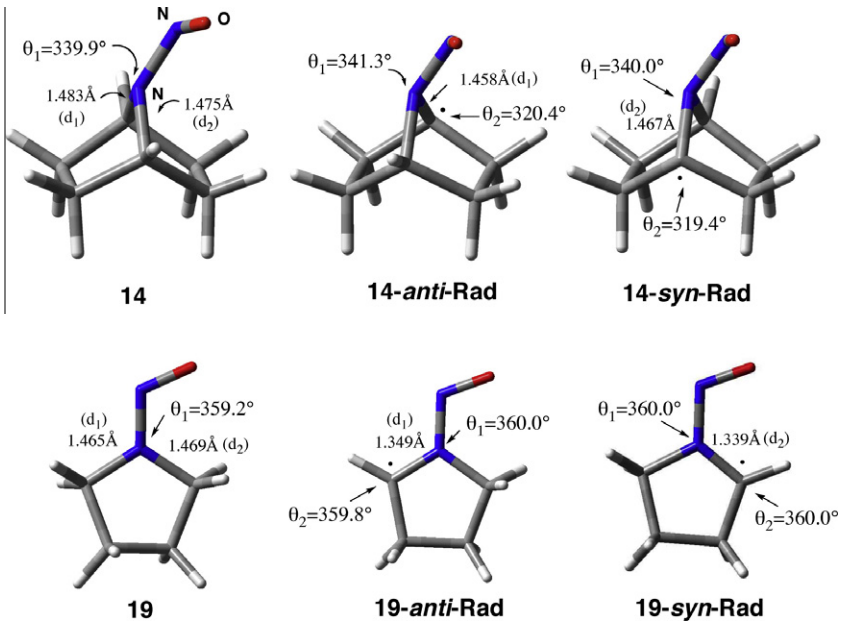
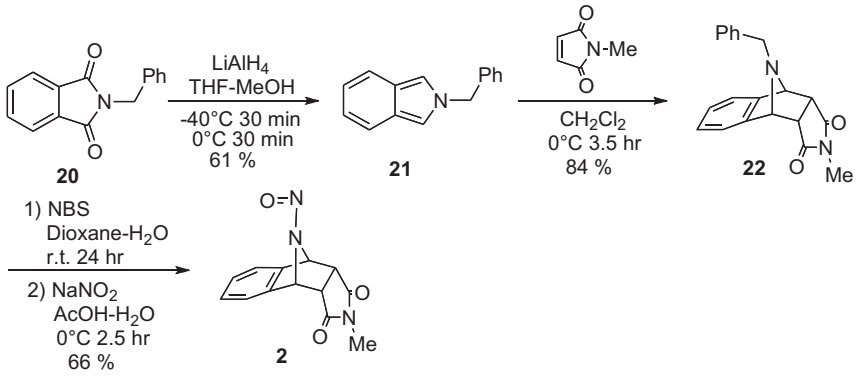


Figure 7. Structural features of nitrosamines and the corresponding carbon radical species. θ_1 and θ_2 are the summation of the three bond angles of the nitrogen and radical carbon atoms, respectively. d_1 and d_2 are the bond lengths between the nitrogen and the relevant carbon atoms, respectively.

TA98. Since the presence in *Salmonella* of the *R* factor, pKM101, which is believed to enhance error-prone DNA repair[37] enhances the number of revertants induced by *N*-nitrosodialkylamines,[38,39] the mutagenicity of bicyclic nitrosamines was determined with the University of Tokyo. Compounds **1**–**3**, **6**–**8**, **14**, **15**, **18** and **19** were synthesized as previously described.[13,33]

4.3.2. Synthesis of 2



S. typhimurium TA100 and TA98 for detection of base substitution and frame-shift mutation, respectively.

4.2. Ames assay

The bacterial mutation assay was carried out according to the pre-incubation method reported by Ames.[15] Test compounds were diluted to the concentrations of 0.03, 0.06, 0.13, 0.25, 0.5 mg/50 μL DMSO. The DMSO solution at each concentration was put into a test tube, and then 0.5 mL of S9 mix or 0.1 M sodium phosphate buffer (pH 7.4) was added, followed by 0.1 mL of a culture of the tester strain. The mixture was incubated for 30 min at 37 °C with shaking (60 strokes/min), and then top agar (2 mL) was added. The mixture was poured onto a minimal-glucose agar plate. After incubation at 37 °C for 44 h, colonies were counted. All plates were prepared in duplicate and the experiments were repeated at least twice. Data are presented as the mean of duplicate determinations. The results were considered positive if the assay produced reproducible, dose-related increases in the number of revertants, while a mutagen was considered weakly positive if it produced a reproducible, dose-related increase in the number of revertants, but the number of revertants was less than double the background number of revertants.[15]

4.3. Synthesis of compounds

4.3.1. General methods

All the melting points were measured with a Yanaco Micro Melting Point Apparatus and are uncorrected. Proton (400 MHz) NMR spectra were measured on a Bruker Avance 400 NMR spectrometer with TMS as an internal reference in $CDCl_3$ as the solvent, unless otherwise specified. Chemical shifts δ are shown in ppm. Coupling constants are given in hertz. Low-resolution electron-impact (EI) mass spectra (MS, EI+) and high-resolution EI mass spectra (HRMS, EI+) were recorded on a JEOL JMS-O1SG-2 spectrometer and on a JEOL JMS-SX 102A. Low-resolution FAB mass spectra (MS, FAB+) and high-resolution FAB mass spectra (HRMS, EI+) were recorded on a JEOL MStation JMS-700 spectrometer and on a JEOL JMS-SX 102A. Electron-spray ionization time-of-flight mass spectra (ESI-TOF MS, ESI+) were recorded on a Bruker Daltonics, micro-TOF05. The combustion analyses were carried out in the microanalytical laboratory of Graduate School of Pharmaceutical Sciences,

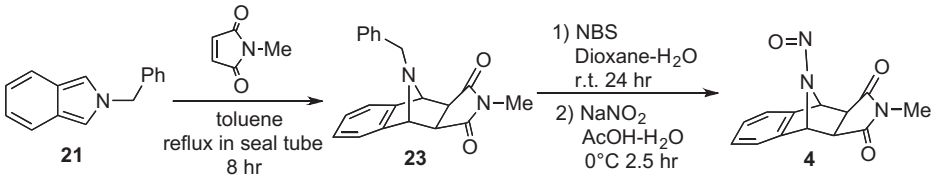
N-Benzylisoindole (**21**): To a suspension of $LiAlH_4$ (3.61 g, 94.9 mmol) in 75 mL of dry THF under Ar, a solution of 6.5 mL of methanol in 75 mL of THF was added over 30 min at 0 °C, followed by cooling of the whole to −78 °C (in an acetone-dry ice bath). To this suspension, *N*-benzylphthalimide **20** (7.23 g, 30.5 mmol) was added in portions at −78 °C, and the mixture was stirred at this temperature for 30 min. Then the mixture was stirred at 0 °C for another 30 min. A solution of sodium sulfate (1.0 g) in 9 mL of water was added, and the resultant inorganic salt was filtered by suction, and the precipitate was washed with acetone. The combined organic layer was dried over sodium sulfate, and the organic solvent was evaporated below 40 °C. To the obtained residue, 20 mL of ethanol was added, and the resultant suspension was allowed to stand in refrigerator for overnight. The precipitate was collected and washed with cold ethanol, affording 3.86 g (18.6 mmol) of *N*-benzylisoindole **21** (61% yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.52 (2H, m), 7.31 (3H, m), 7.14 (4H, m), 6.92 (2H, m), 5.36 (2H, m).

Compound **22**: To a solution of *N*-benzylisoindole **21** (1.12 g, 5.31 mmol) in 15 mL of CH_2Cl_2, a solution of *N*-phenylmaleimide (613 mg, 5.52 mmol) in 3 mL of CH_2Cl_2 was added over 5 min at 0 °C. The whole was stirred at 0 °C for 2.5 h. The residue, obtained after evaporation of the solvent, was column-chromatographed (*n*-hexane/ethyl acetate = 3:1) to give the adduct **22** (1.42 g, yield 84%). MS ($[M+H]^+$): 319. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.33–7.18 (9H, m), 4.57 (2H, m), 3.69 (2H, m), 3.38 (2H, m), 2.26 (3H, s).

Nitrosamine **2**: To a solution of the adduct **22** (1.42 g, 4.46 mmol) in 20 mL of dioxane, a solution of NBS (957 mg, 5.35 mmol) in 8 mL of H_2O was added over 2 min at ambient temperature. The mixture was stirred at room temperature for 18 h. The residue, obtained after evaporation of the solvents, was dissolved in 8 mL of acetic acid and 18 mL of water. To this solution, an aqueous solution of $NaNO_2$ (415 mg, 6.01 mmol) in 10 mL of water was added over 5 min at 0 °C. The whole was stirred at 0 °C for 2.5 h, then extracted with $CHCl_3$, and the organic layer was washed with saturated aqueous Na_2CO_3, brine and dried over Na_2SO_4. The organic solvent was evaporated to give a residue, which was column-chromatographed (*n*-hexane/AcOEt = 2:1) to give **2** (758 mg, 2.94 mmol, 55% yield). Mp; 139–140 °C (yellow powder, recrystallized from *n*-hexane/CH_2Cl_2). HRMS ($[M+H]^+$): Calcd for $C_{13}H_{10}N_3O_4^+$: 257.0873. Found: 258.0875. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.44–7.24 (4H, m), 6.44–6.12 (2H, m),

3.84–3.52 (2H, m), 2.33 (3H, m). Anal. Calcd for $C_{13}H_{11}N_2{}^{15}N_1O_3$ (^{15}NO): C, 60.46; H, 4.29; N, 16.66. Found: C, 60.63; H, 4.45; N, 16.40.

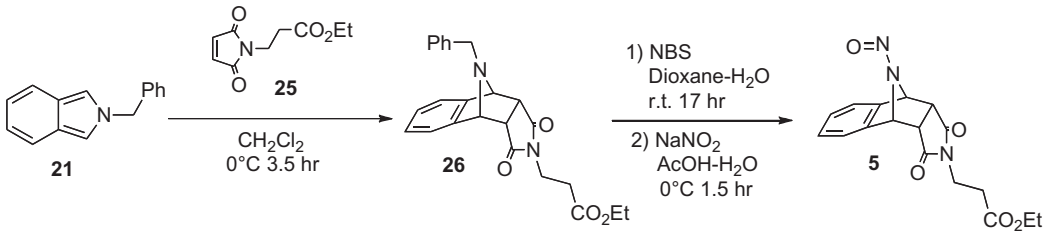
4.3.3. Synthesis of 4



Compound **23**: The preparation of **23** was carried out in a similar manner to **22**. White powder (1.38 g, 52%). MS ([M+H]$^+$): 319. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.36–7.34 (2H, m), 7.28–7.21 (5H, m), 7.04–7.01 (2H, m), 4.50 (2H, s), 3.27 (2H, s), 3.10 (3H, s), 2.82 (2H, s).

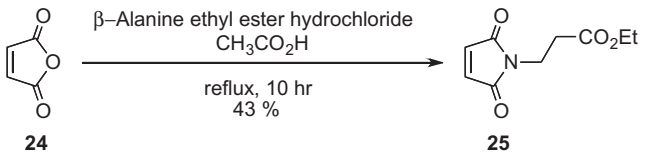
Nitrosamine **4**: The preparation of **4** was carried out in a similar manner to **2**. Mp: 163–167 °C (yellow powder, recrystallized from methanol). MS ([M+H]$^+$): 258. Anal. Calcd for $C_{13}H_{11}N_3O_3$: C, 60.70; H, 4.31; N, 16.33. Found: C, 60.55; H, 4.43; N, 16.12. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.51–7.33 (2H, m), 7.30–7.27 (2H, m), 6.38 (1H, br s), 6.18 (1H, br s), 3.18 (1H, br s), 3.04 (1H, br s), 2.96 (3H, m).

Compound **26**: The preparation of **26** was carried out in a similar manner to **22**. MS m/z: 405 ([M+H]$^+$). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.33–7.18 (9H, m), 4.58 (2H, br s), 4.04 (2H, q, J = 7.2 Hz), 3.70 (2H, br s), 3.36 (2H, br s), 3.18 (2H, t, J = 8.0 Hz), 1.62 (2H, t, J = 8.0 Hz), 1.20 (3H, t, J = 7.2 Hz).



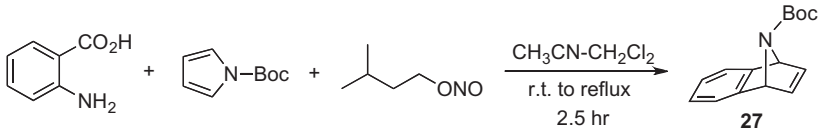
Nitrosamine **5**: The preparation of **5** was carried out in a similar manner to **2**. Mp: 59.3–61.0 °C (yellow plates, recrystallized from *n*-hexane/CH_2Cl_2). Anal. Calcd for $C_{17}H_{17}N_3O_5$: C, 59.47; H, 4.99; N, 12.24. Found: C, 59.40; H, 5.01; N, 12.08. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.41–7.26 (4H, m), 6.40–6.13 (2H, m), 4.06 (2H, q, J = 7.2 Hz), 3.84–3.61 (2H, m), 3.22 (2H, t, J = 8.0 Hz), 1.67 (2H, t, J = 8.0 Hz), 1.21 (3H, t, J = 7.2 Hz).

4.3.4. Synthesis of 5



N-(Ethyl propionlate)imide **25**: The synthesis of **25** was described previously.[40] To a solution of maleic anhydride **24** (480 mg, 4.89 mmol) in 8 mL of glacial acetic acid, β-alanine ethyl ester hydrochloride (800 mg, 5.21 mmol) was added in portions at ambient temperature and the mixture was refluxed for 10 h. The whole was extracted with $CHCl_3$, and the organic layer was washed with 2 N aqueous NaOH and brine, dried over sodium sulfate. The organic solvent was evaporated to give **25** as a colorless oil (412 mg, 2.09 mmol, 43% yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ 6.77 (2H, s), 4.13 (2H, q, J = 7.2 Hz), 3.82 (2H, t, J = 7.2 Hz), 2.63 (2H, t, J = 7.2 Hz), 1.25 (3H, t, J = 7.2 Hz).

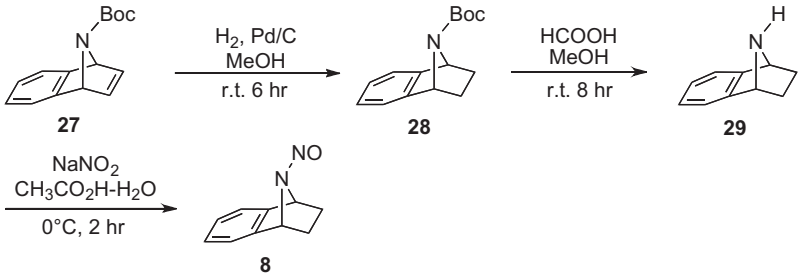
4.3.5. Synthesis of 8



Compound **27**: To a solution of *N*-Boc pyrrole (1.50 g, 9.0 mmol) in 60 mL of CH_3CN was added a solution of anthranilic acid (1.03 g, 7.5 mmol) in 35 mL of CH_2Cl_2 and a solution of isoamyl nitrite (1.76 g, 15 mmol) in 30 mL of CH_3CN simultaneously at 25 °C over 30 min. The mixture was refluxed for 2 h, then the whole was cooled to rt. The organic solvents were evaporated to give a reddish

brown oil, which was column-chromatographed (Et_2O/*n*-hexane = 1:3 to 1:2) to give **27** (924 mg, 41%) as a brown oil. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.26–7.25 (2H, br m), 7.03–6.93 (4H, br m), 5.45 (2H, br s), 1.38 (9H, s).

Compound **30**: The preparation of **30** was carried out in a similar manner to **27**. Brown oil (1.58 g, yield 50%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.11–6.91 (4H, m), 5.52–5.39 (2H, m), 3.84 (6H, s), 1.38 (9H, s).



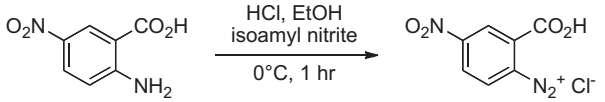
Compound **28**: The alkene derivative **27** (1.33 g, 5.47 mmol) was hydrogenated over 10% Pd–C (148 mg) in methanol for 6 h at ambient temperature. The reaction mixture was filtrated and the solvent was evaporated to give **28** as a pale yellow oil. (1.22 g, 91% yield). MS m/z: 246 ([M+H]$^+$). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.24–7.21 (2H, d,d, *J* = 3.2 Hz, 5.3 Hz), 7.14–7.11 (2H, d,d, *J* = 3.2 Hz, 5.3 Hz), 5.10 (2H, s), 2.16–2.02 (2H, m), 1.39 (9H, s), 1.30–1.26 (2H, m).

Nitrosamine **8**: Compound **28** (1.52 g, 6.21 mmol) was dissolved in 10 mL of formic acid and the mixture was stirred for 8 h at room temperature under argon. The solvent was evaporated and the residue was washed with 10% aqueous Na_2CO_3 and the whole was extracted with CH_2Cl_2. The organic layer was dried over sodium sulfate, and evaporated to give the crude of amine derivative **29** (839.6 mg, 5.79 mmol). To a solution of the amine derivative **29** (839.6 mg, 5.79 mmol) in a mixture of 3.8 mL of acetic acid and 5.0 mL of water, an aqueous solution of $NaNO_2$ (500 mg, 7.24 mmol) in 8.0 mL of H_2O was added over 5 min at 0 °C (in an ice-water bath), and the whole was stirred for 2 h. The whole was extracted with $CHCl_3$, and the organic layer was washed with brine, and dried over sodium sulfate. The organic solvent was evaporated to give a residue, which was column-chromatographed (AcOEt/*n*-hexane = 2:5) to give a yellow solid **8** (517 mg, 51%). Mp; 59.3–61.0 °C (yellow plates, recrystallized from *n*-hexane/CH_2Cl_2). MS (M+H)$^+$: 175.1. Anal. Calcd for $C_{10}H_{10}N_2O$: C, 68.95; H, 5.79; N, 16.08. Found: C, 69.18; H, 5.63; N, 16.03. ^{1}H NMR ($CDCl_3$/TMS): δ 7.37–7.21 (4H, m), 5.94–5.91 (2H, m), 2.29–2.01 (2H, m), 1.61–1.35 (2H, m).

31: The preparation of **31** was carried out in a similar manner to **28**. Yield 81%. MS (M+H)$^+$: 306. ^{1}H NMR (400 MHz, $CDCl_3$): δ 6.86 (2H, s), 5.05 (2H, s), 3.86 (6H, s), 2.07 (2H, m), 1.40 (9H, s), 1.23 (2H, m).

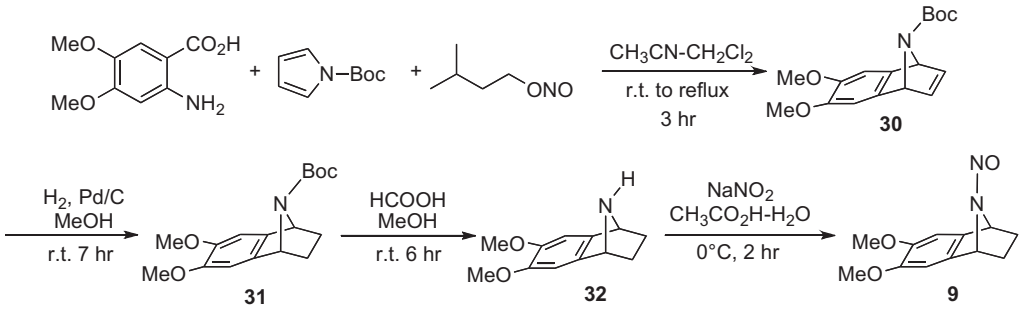
Nitrosamine **9**: The preparation of **9** was carried out in a similar manner to **8**. Mp: 161–164 °C (pale yellow plates, recrystallized from THF/*n*-hexane). Yield 38%. MS (FAB, [(M+H)$^+$]): 235.1. Anal. Calcd for $C_{12}H_{14}N_2O_3$: C, 61.53; H, 6.02; N, 11.96. Found: C, 61.61; H, 6.03; N, 11.94. ^{1}H NMR (400 MHz, $CDCl_3$): δ 6.96–6.87 (2H, m), 5.87–5.85 (2H, m), 3.89 (3H, s), 3.87 (3H, s), 2.25–2.17 (1H, m), 2.05–1.98 (1H, m), 1.56–1.51 (1H, m), 1.37–1.33 (1H, m).

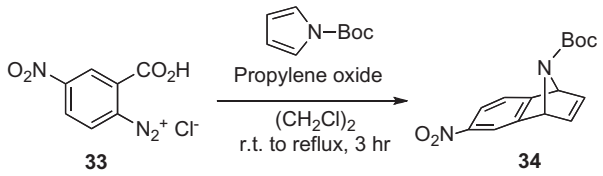
4.3.7. Synthesis of 10



Compound **33**: To a solution of 5.0 g (27.5 mmol) of 5-nitroanthranilic acid in ethanol (75 mL) was added slowly 2.75 mL of concentrated hydrochloric acid at 0 °C. The resulting clear solution was kept below at 0 °C and then 5.5 mL of isoamyl nitrite was added dropwise (15 min). After the solution was stirred at 0 °C for 1 h, 85 mL of ether was added, and the mixture was stirred for another 1 h at 0 °C, during which an orange precipitate was formed. The product was collected and washed with ether and dried under vacuum at rt, yield 3.24 g (51%).

4.3.6. Synthesis of 9

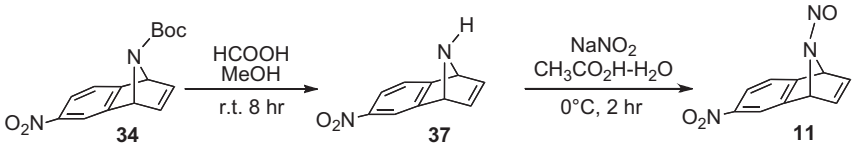




Nitrosamine **10**: The preparation of **10** was carried out in a similar manner to **8**.

Yellow solid (112 mg, 28.5% (from **35**)). HRMS ([M+H]$^+$): Calcd for $C_{10}H_{10}N_3O_3$: 220.0723. Found: 220.0718. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.68–8.63 (1H, m), 8.34–8.37 (1H, m), 7.66–7.64 (1H, m), 5.97–5.88 (2H, m), 2.33–2.11 (1H, m), 2.11–2.00 (1H, m), 1.66–1.54 (1H, m), 1.45–1.34 (1H, m).

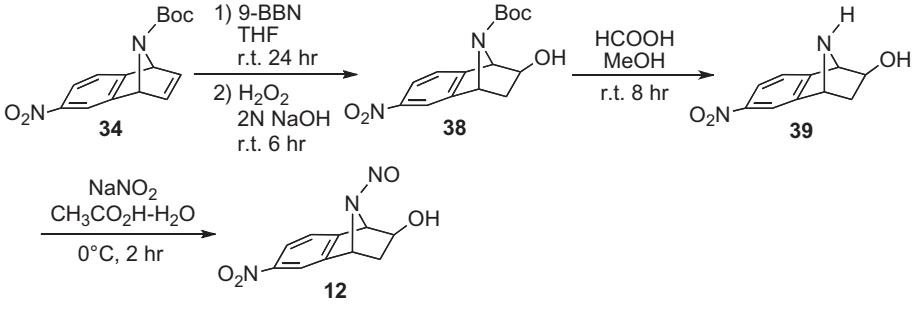
4.3.8. Synthesis of 11

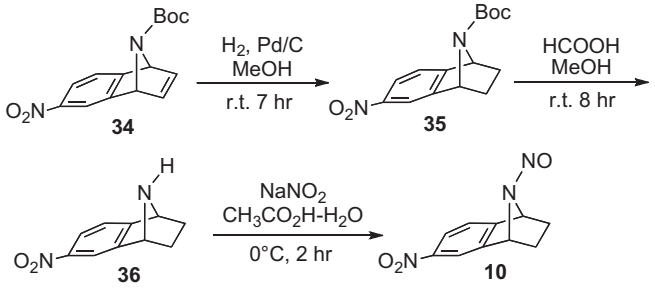


Compound **34**: To a solution of *N*-Boc-pyrrole (2.72 g, 16.3 mmol) in ethylene dichloride (20 mL), a solution of 2-carboxy-4-nitro-benzenediazonium chloride **33** (3.74 g, 16.3 mmol) in 20 mL of ethylene dichloride was added at ambient temperature. To this stirred solution 3 mL of propyleneoxide in 10 mL of ethylene dichloride was added and then the solution was gradually warmed. As gas evolution commenced, the temperature was controlled to prevent vigorous gas foaming. After 10 min of gas evolution the solution became clear. The whole was heated at reflux for 3 h, the organic solvent was removed under vacuum, and the brown liquid residue was taken up in ether, washed with aqueous sodium hydroxide and water, and dried over sodium sulfate. The solvent was removed at reduced pressure and the residue was column-chromatographed (Et_2O/*n*-hexane = 1:3 to 1:2) to give **34** (871 mg, 3.02 mmol, 19%) as a reddish-brawn oil. HRMS ([M+Na]$^+$): Calcd for $C_{15}H_{16}N_2O_4Na$: 311.1008. Found: 311.1005. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.06 (1H, d, J = 2.02 Hz), 7.97–7.94 (1H, d, d, J = 7.88 Hz, 2.02 Hz), 7.37 (1H, d, J = 7.88 Hz), 7.01 (2H, d, J = 16.3 Hz), 5.58 (2H, s), 1.38 (9H, s).

Compound **34** (443 mg, 1.54 mmol) was dissolved in formic acid (8 mL). The reaction mixture was stirred for 8 h at rt (under Ar). The solvent was then evaporated and the residue was washed with 10% aqueous Na_2CO_3 and extracted with CH_2Cl_2. The organic phase was dried over Na_2SO_4 and evaporated to afford crude **37** (185 mg, 0.99 mmol). To a solution of the amine derivative **37** (185 mg, 0.99 mmol) in a mixture of 1.2 mL of acetic acid and 1.5 mL of water, an aqueous solution of $NaNO_2$ (152 mg, 2.20 mmol) in 1.2 mL of H_2O was added over 2 min at 0 °C (in an ice-water bath), and the whole was stirred for 1 h. The whole was extracted with $CHCl_3$, and the organic layer was washed with brine, and dried over sodium sulfate. The organic solvent was evaporated to give a residue, which was column-chromatographed (AcOEt/*n*-hexane = 2:5 to 2:3, gradient elution) to give **11** (134.6 mg, 40% from **34**) as an oil. MS (FAB, [(M+H)$^+$]): 218.1. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.32–8.24 (1H, m), 8.06–8.00 (1H, m), 7.76–7.65 (1H, s), 7.39–7.65 (2H, m), 6.94 (1H, s), 6.48 (1H, m).

4.3.9. Synthesis of 12



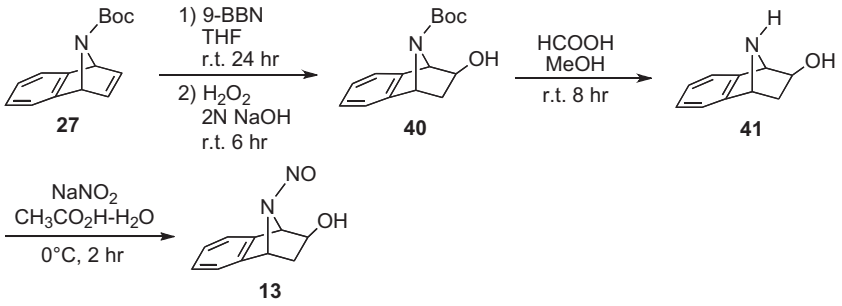


Compound **38**: The preparation of **38** was carried out in a similar manner to **40**. Pale orange powder (yield 50%). HRMS ([M+Na]$^+$): Calcd for $C_{15}H_{18}N_2NaO_5$: 329.1108. Found: 329.1113. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.15–8.14 (1H, m), 8.12–8.10 (1H, m), 7.37–7.35 (1H, m), 5.22–5.21 (1H, m), 5.11 (1H, br s), 1.97–1.87 (1H, m), 1.39 (9H, s), 1.26–1.21 (1H, m).

Nitrosamine **12**: The preparation of **12** was carried out in a similar manner to **13**. Yield 45%. Mp: 151–153 °C (black powder, recrystallized from Et_2O/*n*-hexane). Anal. Calcd for $C_{10}H_9N_3O_4$: C, 51.07; H, 3.86; N, 17.87. Found: C, 50.96; H, 4.01; N, 17.64. ^{1}H NMR (400 MHz, DMSO): δ 8.41–8.11 (2H, m), 7.82–7.61 (1H, m),

6.33–5.63 (3H, m), 4.11–3.95 (1H, m), 2.10–1.81 (1H, m), 1.60–1.54 (1H, m).

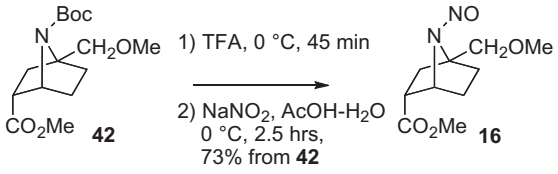
4.3.10. Synthesis of 13



Compound **40**: 9-BBN solution in THF (0.4 M, 32.3 mL, 12.9 mmol) was added to alkene **27** (2.61 g, 10.8 mmol) over 5 min at rt under argon. The mixture was stirred for 24 h at rt, then cooled to 0 °C. H_2O_2 (13.5 mL of a 35% aqueous solution) was added dropwise, followed by NaOH (7.8 mL of a 2 N aqueous solution). The whole was allowed to warm to 25 °C and stirred for 6 h. The aqueous layer was saturated with K_2CO_3, and the organic layer was extracted with Et_2O, dried over sodium sulfate. The solvent was evaporated to give a residue, which was column-chromatographed (Et_2O/*n*-hexane = 1:1 to 2:1) to give **40** as pale yellow oil (2.08 g, yield 74%). MS $(M+H)^+$: 262. ^1H NMR (400 MHz,$CDCl_3$): δ 7.27–7.23 (2H, m), 7.19–7.17 (2H, m), 5.11 (1H, s), 5.01 (1H, s), 4.04–4.00 (1H, m), 3.20 (1H, br s), 1.87–1.84 (2H, m), 1.38 (9H, s).

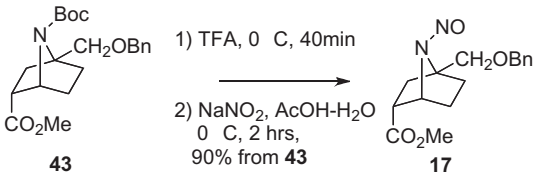
Nitrosamine **13**: Compound **40** (1.24 g, 4.74 mmol) was dissolved in a mixture of formic acid (10 mL) and methanol (5 mL) and the reaction mixture was stirred for 8 h at room temperature under argon atmosphere. Then the solvent was evaporated and the whole was washed with 10% aqueous Na_2CO_3 and the residue was extracted with CH_2Cl_2. The solvent was dried over sodium sulfate and evaporated to give crude **41** (735 mg, 4.57 mmol). To a solution of the amine derivative **41** (735 mg, 4.57 mmol) in a mixture of 3.0 mL of acetic acid and 8.0 mL of H_2O, an aqueous solution of $NaNO_2$ (434 mg, 6.29 mmol) in 4.0 mL of H_2O was added over 5 min at 0 °C, and the whole was stirred for 2 h. The reaction mixture was extracted with $CHCl_3$, and the organic layer was washed with brine, and dried over sodium sulfate. The organic solvent was evaporated to give a residue which was chromatographed (AcOEt/*n*-hexane = 1:2 to 1:1) to give **13** (535 mg, yield 59% from **40**). Mp; 74.0–77.0 °C (pale black plates, recrystallized from *n*-hexane/Et_2O). HRMS (ESI, $[M+H]^+$): Calcd for $C_{10}H_{11}N_2O_2$: 191.0821. Found: 191.0822. Anal. Calcd for $C_{10}H_{10}N_2O_2$: C, 63.15; H, 5.30; N, 14.73. Found: C, 63.25; H, 5.30; N, 14.62. ^1H NMR (400 MHz, $CDCl_3$): δ 7.42–7.17 (4H, m), 5.96–5.75 (2H, m), 4.26–4.15 (1H, m), 2.18–2.10 (1H, m), 2.00–1.95 (1H, m), 1.79–1.75 (1H, m).

4.3.11. Synthesis of 16



Nitrosamine **16**: Compound **42** (86.8 mg, 0.2900 mmol)[42] was dissolved in TFA 1 mL at 0 °C and the reaction mixture was stirred for 45 min at this temperature. Then TFA was evaporated to give the crude amine. To a solution of the resultant amine derivative in 1 mL of acetic acid, an aqueous solution of $NaNO_2$ (74.7 mg, 1.083 mmol) in 2 mL of H_2O was added over 1 min at 0 °C, and the whole was stirred for 2.5 h at this temperature. The reaction mixture was poured into water (50 mL) and extracted with Et_2O (25 mL × 3). The organic layer was washed with water and brine (5 mL). The organic layer was dried over Na_2SO_4 and evaporated. Purification by column-chromatography (*n*-hexane/AcOEt = 3:1) gave compound **16** (48.6 mg, 0.2129 mmol, 73% yield) as a yellow oil. ^1H NMR ($CDCl_3$): δ = 5.21–5.18 (1H, m), 4.12 (1H, d, *J* = 10.4 Hz), 4.09 (1H, d, *J* = 10.8 Hz), 3.73 (3H, s), 3.48 (3H, s), 3.03–2.99 (1H, m), 2.25–1.63 (6H, m). ^{13}C NMR ($CDCl_3$): δ =171.76, 70.26, 69.53, 59.65, 54.40, 52.28, 43.15, 35.09, 31.89, 23.02. HRMS (ESI-TOF, $[M+Na]^+$): Calcd for $C_{10}H_{16}N_2NaO_4^+$, 251.1002. Found: 251.1001. Anal. Calcd for $C_{10}H_{16}N_2O_4$: C, 52.62; H, 7.07; N, 12.27. Found: C, 52.62; H, 6.91; N, 12.27.

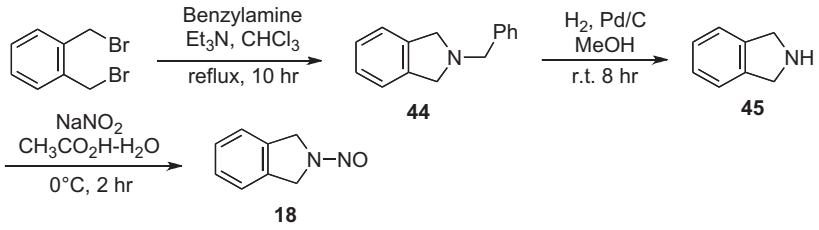
4.3.12. Synthesis of 17



Nitosamine **17**: Compound **43** (74.7 mg, 0.1990 mmol)[41] was dissolved in TFA 1.0 mL at 0 °C and the reaction mixture was stirred for 40 min at this temperature. Then TFA was evaporated and to give the crude amine. To a solution of the resultant amine derivative in 0.5 mL of acetic acid, an aqueous solution of $NaNO_2$ (60.0 mg, 0.8696 mmol) in 1.0 mL of H_2O was added over 1 min at 0 °C, and the whole was stirred for 2 h at this temperature. The reaction mixture was poured into water (40 mL) and extracted with Et_2O (30 mL × 3). The organic layer was washed with brine (5 mL). The organic layer was dried over Na_2SO_4 and evaporated. Purification by column-chromatography (*n*-hexane/AcOEt = 3:1) gave compound **17** (54.5 mg, 0.1791 mmol, 90% from **43**) as a yellow oil. ^1H NMR ($CDCl_3$): δ = 7.36–7.30 (5H, m), 5.21 (1H, m), 4.66 (2H, s), 4.20 (1H, d, *J* = 10.8 Hz), 4.16 (1H, d, *J* = 10.4 Hz), 3.73 (3H, s), 3.02–2.99 (1H, m), 2.28–1.62 (6H, m). ^{13}C NMR ($CDCl_3$): δ = 171.68, 137.74, 128.32, 127.67, 127.55, 73.58, 69.52, 67.71, 54.35, 52.20, 43.09, 35.15, 31.97, 22.96. HRMS (ESI-TOF, $[M+Na]^+$): Calcd for $C_{16}H_{20}N_2NaO_4^+$, 327.1321. Found: 327.1309. Anal. Calcd for $C_{16}H_{20}N_2O_4$: C, 63.14; H, 6.62; N, 9.20. Found: C, 63.00; H, 6.60; N, 9.12.

4.3.13. Synthesis of *N*-nitrosoisoindoline 18

The synthesis of **18** was carried out as described previously (Ref. 33).



Isoindoline **45**: To a solution of α,α-dibromo-*o*-xylene (5.23 g, 19.9 mmol) in $CHCl_3$ (15 mL), a solution of triethylamine (6 mL) in $CHCl_3$ (10 mL) was added at 0 °C over 5 min. To this solution, benzylamine (2.21 g, 20.6 mmol) in 10 mL of $CHCl_3$ was added at 0 °C over 5 min. The whole was heated at reflux for 10 h and the solvent was evaporated to give a residue (**44**). The *N*-benzyl derivative **44** was hydrogenated over 10% Pd–C (420 mg) in methanol (15 mL) for 8 h at ambient temperature. The reaction mixture was filtrated to remove Pd–C and the solvent was evaporated to give amine derivative **45**. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.43–7.13 (9H, m), 3.91 (4H, s), 3.88 (2H, s). MS ($[M+H]^+$): 210.

N-Nitrosoisoindoline **18**: To a solution of isoindoline **45** in a mixture of 10 mL of acetic acid and 20 mL of water, an aqueous solution of $NaNO_2$ (1.53 g, 22.2 mmol) in 10 mL of water was added at 0 °C. The whole was stirred for 2 h, then extracted with CH_2Cl_2, and the organic layer was washed with 2 N aqueous NaOH, 0.5 N aqueous HCl, brine, and dried over sodium sulfate. The solvent was evaporated to give a residue, which was chromatographed (hexane: AcOEt = 1: 4 to 1: 3) to give **18** as an oil (1.32 g, 8.91 mmol, 45% from α,α-dibromo-*o*-xylene). Mp: 85–89 °C (recrystallized from MeOH, grayish needles). MS ($[M+H]^+$): 149. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.40–7.31 (4H, m), 5.63 (2H, s), 4.90 (2H, s).

N-Nitrosopyrrolidine (**19**): *N*-nitrosopyrrolidine (**19**) was synthesized by nitrosation of pyrrolidine and purified by column-chromatography as described previously.[33] Further purification was carried out in the present study: the synthesized *N*-nitrosopyrrolidine (**19**) was dissolved in methanol saturated with sodium hydroxide and the whole solution was stirred overnight at room temperature, then purified by extraction (see Ref. 18d).

4.4. Calculations

All conformations of each molecule were optimized in the Gaussian 03 program[42] at the B3LYP/6-31G(d) (method A) or CBS-Q level (method D) of theory to find the global minimum. The number of imaginary frequencies confirmed ground states. Zero-point energy corrections were applied without scaling. Single-point energies at 273.15 K were obtained by two DFT methods, R(U)B3LYP/6-311++G(d,p) (method B) and R(U)B3P86/6-311++G(d,p) (method C) on the basis of the R(U)B3LYP/6-31G(d)-optimized structures. The lowest-energy structure of **9** with respect to two methoxy groups was obtained with MacroModel 8.1 programs; Monte Carlo (MC) conformational search was performed using the OPLS-AA force field in the gas phase. One thousand starting structures were generated. The lowest-energy conformation with respect to the two methoxy groups was used for the DFT calculations.

Acknowledgments

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