Generation, basic chemistry, and detection of *N*-nitrosotryptophan derivatives

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N-Terminal blocked tryptophan derivatives like melatonin or tryptophan residues in peptides are easily nitrosated at the nitrogen atom of the indole ring to give the corresponding *N*-nitrosotryptophan derivatives. This article provides a comprehensive view of the synthesis, chemical properties, and detection methods of this class of *N*-nitroso compounds of potential importance in biological systems.

Introduction

In 1901 Hopkins and Cole[1] were the first to isolate tryptophan, the structure of which was established in 1908 by Ellinger and Flamand.[2] Although several other amino acids had been described before, tryptophan was the first one that was demonstrated to be indispensible.[3,4] Tryptophan is probably the indole derivative most widely distributed in nature. It is converted into many compounds of important biological significance, *e.g.* neurohormones (serotonin and melatonin), the phytohormone indoleacetic acid, some pigments found in the eyes of insects, and a variety of alkaloids.[5]

In chemical systems, *N*-terminal blocked tryptophan derivatives like *N*-acetyltryptophan (but not tryptophan itself) can be easily nitrosated at the nitrogen atom of the indole ring even at physiological pH values.[6] This capability is very similar to the nitrosation of cysteine and its derivatives to give the corresponding *S*-nitrosothiols.[7] It is currently often proposed that the nitrosation of cysteine residues in proteins should be an important posttranslational modification event in signal transduction mechanisms as well as in the storage and transport of nitric oxide.[8–10] Since N_2O_3 reacts at physiological pH with *N*-terminal blocked tryptophan derivatives as fast as with cysteine residues,[6] *N*-nitrosotryptophan residues must also be expected to be produced endogenously. It has been demonstrated in experimental systems that pharmacological effects of *N*-nitrosotryptophan compounds like vasorelaxation and platelet inhibiting activity are comparable to the ones induced by *S*-nitrosocysteine derivatives.[11] In contrast to *S*-nitrosothiols, *N*-nitrosotryptophan derivatives react spontaneously with various biologically important molecules at physiological pH. During such reactions nitric oxide is often released. The strongly underestimated capability to release nitric oxide suggests that *N*-nitrosotryptophan derivatives act as special nitric oxide storage compounds. Generally, the understanding of the biological impact of a certain compound or class of compounds can only be developed when its basic chemistry is well understood, when reproducible protocols for its synthesis exist, and when sensitive and versatile detection procedures are available. In order to address

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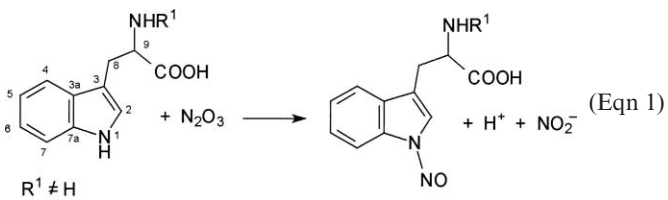
these issues for *N*-nitrosotryptophan derivatives, we studied their reactions with biologically important molecules intensively. The present findings are summarized in this report.

Preparation of *N*-nitrosotryptophan compounds

The traditional synthetic route to *N*-terminal blocked *N*-nitrosotryptophan derivatives is the reaction between tryptophan derivatives and nitrite in acidic media.[12] At acidic conditions (pH $\leq$ 3), nitrite is expected to yield an electrophilic nitrosating intermediate (NO^+, or its equivalent, *e.g.*, $H_2NO_2^+$, N_2O_3)[7] which attacks the five-membered ring of the indole moiety. In indoles with the 3-position unsubstituted, nitrosation takes place very rapidly at C-3 and the thus formed *C*-nitroso compound tautomerizes quickly (where possible) to the oxime form.[13] In 3-substituted tryptophan derivatives the corresponding *N*-nitroso compounds are produced almost quantitatively to give mixtures of the *Z*- and *E*-conformers with respect to the N–N=O group (*ca.* 35% and 65%, respectively), as proven by ^{15}N NMR spectrometry.[6,14–16] The discrimination of the conformers by ^{15}N NMR at room temperature in solution is possible because the rotational barrier of the N–NO bond is relatively high (high level quantum mechanical calculations performed at the CBS-Q3 level of theory predict a barrier of 17.7 kcal mol^{-1} in aqueous solution (calculations not shown) which is about 62% of the N–NO bond dissociation energy[17]). Detailed protocols for the preparation of both *N*-acetyl-*N*-nitrosotryptophan (NANT) and *N*-nitrosomelatonin are given in the literature.[13,15,18]

Some ambiguities exist about the precise mechanism of the nitrosation process. From chemical experience one would expect that electrophilic nitrosating intermediates directly attack the nitrogen atom of the indole ring (eqn (1)):



(Eqn 1)

Somewhat surprisingly, however, with consideration of the molecular structure of 3-substituted indoles, it has been concluded that the most nucleophilic center of the molecule is the C-3 position,[19,20] because in 18 M sulfuric acid protonation occurs at this position.[21] Therefore, it was proposed that *N*-terminal blocked tryptophan derivatives were initially nitrosated at the C-3 position and that the final *N*-nitroso product is formed *via* an internal transfer of the NO^+ function.[19,20] In contrast to this proposal, Hinman and Whipple[21] noted that hydrogen–deuterium exchange of the indole NH (*via* intermediate NHD^+) is strongly accelerated already in 20 μM D_2SO_4, *i.e.*, at a proton concentration that does not lead to protonation at the C-3 position. This fact strongly suggests that the C-3 position does not represent the primary nucleophilic center. In line with this observation, DFT calculations for *N*-acetyltryptophan predict that the largest fraction of negative charge on the indole ring (Mulliken, electrostatic, and natural charge) is located at the N-1 position (Table 1).

Table 1 Calculated charges of the heavy atoms of the indole ring in *N*-acetyltryptophan

	Charge[a]		
Position	Mulliken	Electrostatic	Natural
N-1	−0.704	−0.459	−0.559
C-2	−0.017	−0.083	−0.026
C-3	0.070	−0.116	−0.121
C-4	−0.212	−0.219	−0.222
C-5	−0.140	−0.167	−0.258
C-6	−0.152	−0.085	−0.242
C-7	−0.164	−0.326	−0.265
C-7a	0.318	0.270	0.158
C-3a	0.075	0.092	−0.088

[a] *N*-Acetyltryptophan was optimized at the B3LYP/6-31G(d) level of theory. Charges were calculated at this level by using the Spartan 06 software package.

Thus, for the nitrosation process (eqn (1)) there is no need to assume another nucleophilic center for primary attack other than the secondary nitrogen atom of the indole ring itself.

Interestingly, *N*-terminal blocked tryptophan derivatives can also be smoothly nitrosated at physiological pH values. For this, three nitrosating entities have been suggested. The first nitrosating species is N_2O_3. Recently, two groups reported independently that melatonin is nitrosated by nitric oxide under aerobic conditions.[22,23] The titles of these studies, "Nitrosation of melatonin by nitric oxide"[22] and "Scavenging of NO by melatonin",[23] respectively, appeared to be somewhat misleading with respect to the nitrosating entity because *N*-terminal blocked tryptophan derivatives do not react with nitric oxide in the strict absence of oxygen.[24] However, two well-known N_2O_3-scavengers, morpholine and piperazine,[7] which do not react with *N*-nitrosotryptophan compounds, are highly effective in inhibiting such nitric oxide-dependent nitrosations of *N*-terminal blocked tryptophan derivatives,[6] clearly indicating that under the conditions employed by the two cited papers, N_2O_3 is the actual nitrosating species. From competition experiments with morpholine, rate constants of 4.4×10^7 M^{-1} s^{-1} and 9.2×10^7 M^{-1} s^{-1} were evaluated for the N_2O_3-mediated nitrosation of *N*-acetyltryptophan and melatonin, respectively.[6]

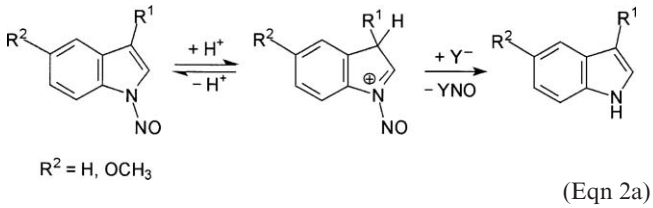
The second compound able to nitrosate *N*-terminal blocked tryptophan derivatives at physiological pH is peroxynitrite.[18,22,25,26] This reaction does not seem to have a decisive impact under physiological situations because in comparison to N_2O_3-generating systems peroxynitrite levels are very low in the (ubiquitous) presence of carbon dioxide.[18,27] Recently, we[28] confirmed a hypothesis of Williams[29] that the peroxynitrite-induced nitrosation of various compounds proceeds *via* N_2O_4, which is somewhat less effective as an electrophilic, amine-nitrosating agent than N_2O_3.[30] In addition, peroxynitrite nitrates melatonin at various positions (N-1, C-4 and C-6).[31] However, nitration at positions C-4 and C-6, but not at N-1, proceeds *via* radical (NO_2) pathways.[32] Thus, the low efficiency of peroxynitrite to *nitrosate* *N*-terminal blocked tryptophan derivatives can be satisfactorily explained with its *nitrating* capabilities.

The third nitrogen–oxygen species capable of producing *N*-nitrosotryptophan compounds is nitroxyl ($HNO/^3NO^-$).[25,33] Mechanistic details of this nitrosation process are so far not fully

understood because of the highly complex chemistry of nitroxyl. Evidence has been found that depending on the actual reaction conditions, a variety of reactive intermediates, such as HO$^{\bullet}$, $^{\bullet}$NO, $O_2^{\bullet-}$, $ONOO^-$, and $HN_2O_2^{\bullet}$, may be produced from nitroxyl.[34–36]

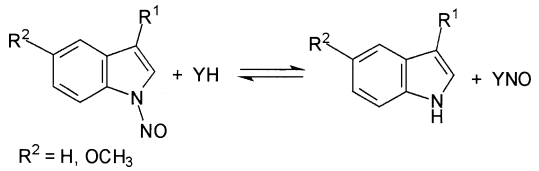
Basic chemistry of *N*-nitrosotryptophan derivatives

In aqueous solution, *N*-nitrosotryptophan derivatives can nitrosate ("transnitrosation") a variety of nucleophiles (Y^-/YH), such as water,[37] halides,[37] pseudo-halides,[37] aliphatic alcohols,[7,38] nucleotides,[39] activated phenols,[40,41] catechols,[42] vitamin C,[6] hydrogen peroxide,[43] thiols,[16] and primary aromatic amines[18] (but not aliphatic secondary ones)[6] at virtually all pH values (0–14) to yield the parent tryptophan derivative and a nitroso compound (YNO). The simplest transnitrosation, *i.e.*, the exchange of the NO^+ function between two *N*-terminal blocked tryptophan derivatives, proceeds even at physiological pH and in non-aqueous solution.[15] The hydrolysis reaction (YH = H_2O) of *N*-nitrosotryptophan derivatives at pH 1–7 is a proton-catalyzed process (eqn (2a)).[37]



(Eqn 2a)

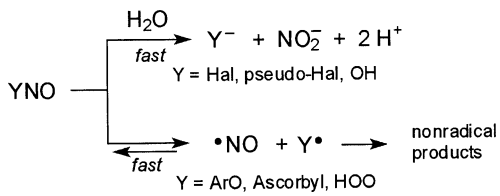
The pH-rate profile of this denitrosation reflects two proton-catalyzed reactions, from which the one dominating in the pH range 4–7 is associated with a halide/pseudohalide nucleophilic catalysis.[37] Although there is no doubt that eqn (2a) is basically correct, it fails to describe the action of some nucleophiles which have been found to denitrosate *N*-nitrosotryptophan derivatives effectively at alkaline pH values. Thus, besides the two proton-catalyzed denitrosation pathways there must be a third, uncatalyzed one in which *N*-nitrosotryptophan derivatives act as electrophilic nitrosating agents, resulting in heterolytic N–NO bond fission (eqn (2b)).



(Eqn 2b)

In accord, it has been shown that various biologically important molecules, for instance, thiols, phenols, and some vitamins, in part react *via* eqn (2b).[16,24,40–42] Since the thus formed nitroso compounds YNO are often very short-lived (preferably because of rapid hydrolysis) and, therefore, may easily escape detection, the relevance of eqn (2b) might be questioned. In fact, de Biase *et al.*[44] proposed that all processes which finally lead to denitrosation of *N*-nitrosotryptophan-derivatives, even the hydrolysis reaction, should proceed *via* initial homolysis of the N–NO bond. However, this assumption cannot be correct, because the aminyl radicals which are initial products of N–NO homolysis are highly unlikely to yield the corresponding amine in stoichiometric amounts in the presence of molecular oxygen. Further, the N–NO bond dissociation energy (28.7 kcal mol^{-1})[17] is too high for a significant contribution of a rate-determining, spontaneous dissociation at ambient temperature. Noticeably, from reaction of NANT with thiols, hydrogen peroxide anion, or melatonin, the corresponding nitroso compounds, *i.e.*, *S*-nitrosothiols, peroxynitrite, and *N*-nitrosomelatonin, respectively, could be identified by ^{15}N-NMR spectrometry.[15,16,43] These observations clearly support a direct reaction as outlined in eqn (2b).

Since the chemical properties of YNO are strongly dependent on the nucleophile part (Y), a variety of products can be generated from *N*-nitrosotryptophan derivatives, depending on the pH value. For example, at pH 4–7 NANT nitrosates halides to yield *N*-acetyltryptophan and the corresponding nitrosyl halides according to eqn (2a).[37] The latter intermediates readily hydrolyze to give nitrous acid ($pK_a(HNO_2) = 3.1$)[45] and the parent halide ion.[7] Thus, in the presence of halides and pseudohalides NANT operates as a clean source for nitrite. A completely different situation is encountered when NANT is reacted with Trolox, a water soluble vitamin E derivative, at pH 7.4–11. In this case the product YNO, an aryl nitrite, is a short-lived species that does not readily hydrolyze but undergoes rapid homolytic dissociation to yield quantitatively nitric oxide and the corresponding Trolox radical. The latter subsequently dismutates (disproportionates) to Trolox and to the related quinone. As no other products were formed in this reaction, NANT now operates as a clean source of nitric oxide. To generalize these observations, homolytic cleavage of the transnitrosation product YNO to produce nitric oxide must always be expected in cases where a stabilized Y$^{\bullet}$ radical will be formed (eqn (3)).



(Eqn 3)

However, the foregoing examples in which the products were generated stoichiometrically are specific cases. More often significantly lower yields of such products are found because the transnitrosation product YNO may react with the educt, as is the case with thiols,[16] or the formed YNO decomposes by various competitive pathways, as is the case with vitamin C,[24] or the *N*-nitrosotryptophan derivative can trap the intermediate reactant radical, as is the case with catechols and vitamin C.[24,42] The most complex situation is encountered when a radical precursor like peroxynitrite[46] is the initial product, as is the case in the NANT–H_2O_2 reaction.[43] Other products from reactions of *N*-nitrosotryptophan derivatives are listed in Table 2.

Quantitative determination of *N*-nitrosotryptophan derivatives

Several procedures have been developed for the determination of *N*-nitrosotryptophan derivatives. In the absence of residues having similar UV–Vis absorptions, the maximums of the electronic absorption around 340 nm ($\varepsilon_{335} = 6100\ M^{-1}\ cm^{-1}$ for NANT[12] and *N*-nitrosotryptophan residues in proteins,[6] $\varepsilon_{346} = 7070\ M^{-1}\ cm^{-1}$ for *N*-nitrosomelatonin[23]) have been used to determine concentrations of *N*-nitrosotryptophan and its derivatives. The moderate extinction coefficients allow the detection of 5–100 μM *N*-nitroso compound in a standard 1 cm cuvette.

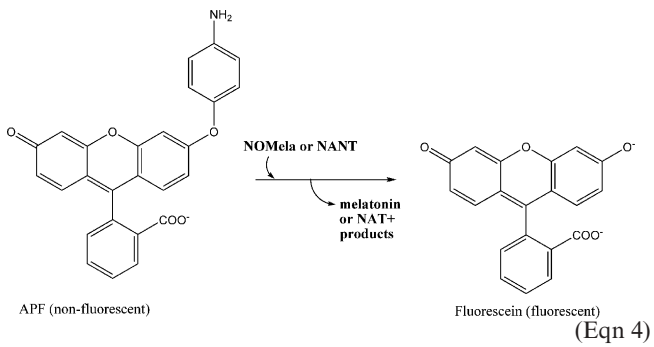
Table 2 Products from reaction of *N*-nitrosotryptophan derivatives with nucleophiles

Reaction	YNO intermediate	Products (yields)	Recommended for detection	Reference
NANT + water	Nitrous acid	Nitrite (100%)	No	37
NANT + halide	Nitrosyl halide	Nitrite (100%)	No	37
NANT + $R^{aliphatic}OH$	Alkylnitrite	Alkylnitrite (n.d.)[a]	No	7,38
NANT + serotonin	Arylnitrite	Nitric oxide (100%)[b]	No	40
NOMela + serotonin	Arylnitrite	Nitric oxide (100%)[b]	No	40
NANT + vitamin C	Alkylnitrite	Nitric oxide (60%), nitroxyl (13%)	No	6
NANT + catechols	Arylnitrite	Various products	No	42
NANT + H_2O_2	Peroxynitrite	Hydroxyl radical, nitrogen dioxide, nitrate, nitrite (pH dependent)	No	43
NANT + Thiol	*S*-Nitrosothiol	*S*-Nitrosothiol (pH dependent)[c]	No	16
NANT + $R_2^{aliphatic}NH$	None	None	No	6
NANT + $R^{aromatic}NH_2$ [d]	Primary aromatic nitrosamines	Triazole (55%), deamination (13%)	Yes, Yes	18
NANT + N_2H_4	*N*-Nitroso-hydrazine	N_2, NH_3, N_2O (n.d.)	No	This work
NANT + MNBDH	*N*-Nitroso-hydrazine derivative	MNBDA (80%)	Yes	This work
NOW[e] + MNBDH	*N*-Nitroso-hydrazine derivative	MNBDA (80%)	Yes	This work
NANT + Trolox	Arylnitrite	Nitric oxide (100%)	Yes	41
NOW[e] + Trolox	Arylnitrite	Nitric oxide (100%)	Yes	41

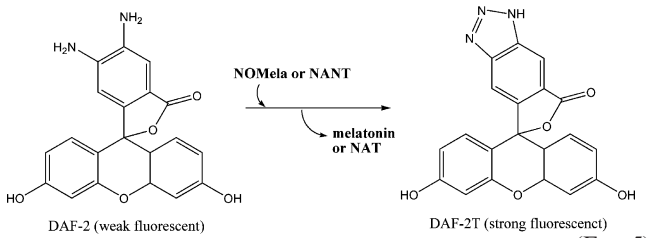
[a] n.d. = not determined. [b] This yield can only be observed at the beginning of the reaction. [c] The yield is decreased by a reaction of the educt with the final product. [d] $R^{aromatic}NH_2$ = aminophenyl fluorescein and 4,5-diaminofluorescein. [e] NOW = *N*-nitrosotryptophan residues in proteins.

In the presence of compounds which absorb in the same wavelength range, similar levels can be detected by separating the *N*-nitrosotryptophan compounds by capillary zone electrophoresis.[15] Somewhat lower detection limits can be achieved (around 1 μM) when the separation process is performed with HPLC.[25,33]

The detection limits can be significantly reduced by employing indirect detection procedures in which the *N*-nitrosotryptophan derivatives, but not *S*-nitrosothiols, react with a suitable compound to produce an easily detectable product. Aromatic amines can be nitrosated by *N*-nitrosotryptophan derivatives[38] to give chemically labile aromatic *N*-nitrosamines.[7,47] Accordingly, the *N*-nitrosamines of aminophenyl fluorescein (APF) and 4,5-diaminofluorescein (DAF-2) decay to give the fluorescent dyes fluorescein and triazolfluorescein (DAF-2T), respectively (eqn (4) and (5)).

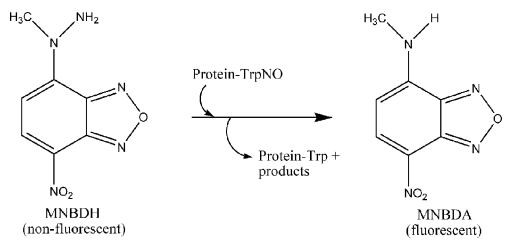


(Eqn 4)



(Eqn 5)

Thus, these aromatic amines can be used as reporter molecules for *N*-nitrosotryptophan derivatives.[18] Although the fluorescent dyes were produced in relatively low yields (55% and 13% in the case of DAF-2 and APF, respectively), the detection limits drop to 100 nM (DAF-2) and 25 nM (APF), respectively. It should be noted, however, that at pH 7.4 the pseudo-first order hydrolysis reaction of *N*-nitrosotryptophan derivatives at concentrations lower than 25 nM is faster than any other transnitrosation reaction.[16] This fact limits the quantification of lower levels of *N*-nitrosotryptophan residues. Unfortunately, the fluorescence of DAF-2T and fluorescein is quenched by present proteins to about 50% and 99%, respectively,[18] so that *N*-nitrosotryptophan residues in proteins cannot be quantified by these methods. In order to improve the detection procedure, other probe systems are required. In 1996 the Stedman[48] group reported that arylhydrazines react effectively with nitrous acid, and in 1999 Büldt and Karst[49] applied the nonfluorescent hydrazine derivative *N*-methyl-4-hydrazino-7-nitrobenzofurazan (MNBDH) for the quantification of nitrite as low as 50 nM in acidic solution. The subsequently generated fluorescent dye *N*-methyl-4-amino-7-nitrobenzofurazan (MNBDA) has a sensitivity limit of 5 nM in non-aqueous solution. A similar reaction between *N*-nitrosotryptophan derivatives and hydrazine compounds, however, has not been reported so far (eqn (6)).



(Eqn 6)

In Fig. 1A it is demonstrated that *N*-acetyl-^{15}*N*-nitrosotryptophan indeed nitrosates $^{15}N_2H_4$ because expected reaction products (*i.e.* $^{15}NH_3$, $^{15}N_2O$, $^{15}N_2$)[50] could be detected by ^{15}N NMR spectrometry.

From this observation it is conceivable that an activated hydrazine compound should react as well, and, in fact, the reaction of NANT with MNBDH generated the fluorescent dye MNBDA with a yield of 80% (Fig. 1B). The major improvement

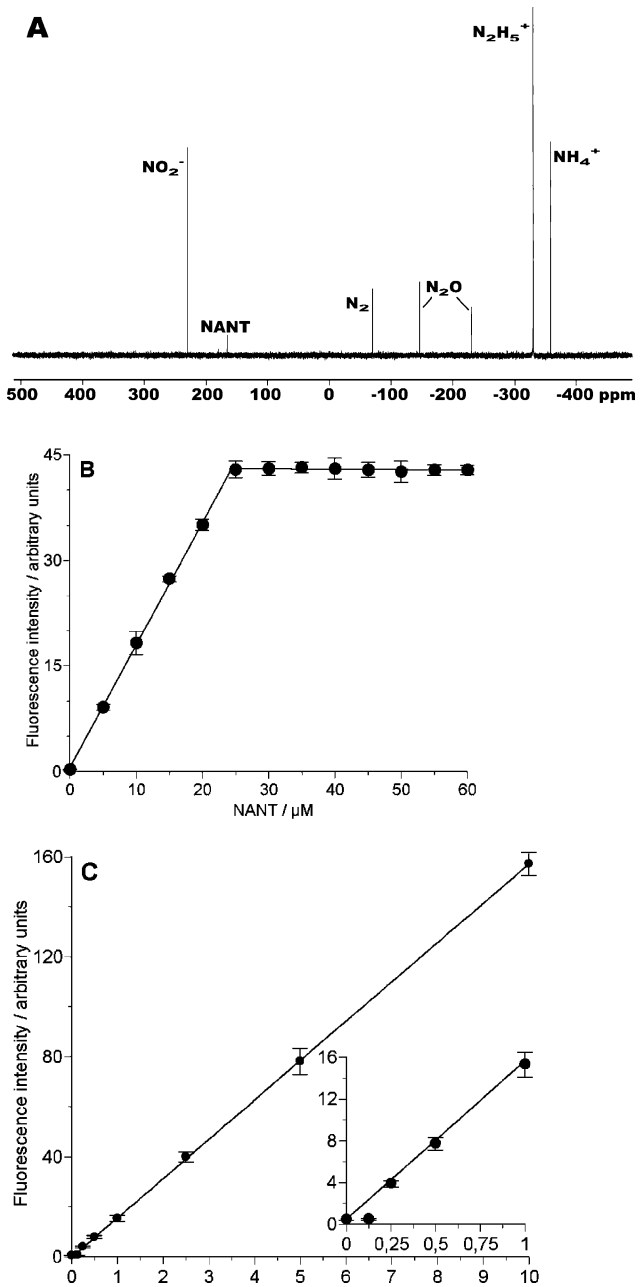


Fig. 1 Evidence that *N*-nitrosotryptophan derivatives can be quantified by using an activated hydrazine derivative. (A) *N*-Acetyl-^{15}*N*-nitrosotryptophan (100 mM) and $^{15}N_2H_4$ (200 mM) were mixed in 100 mM phosphate buffer at pH 7.4 and 25 °C. After 2 h, products were identified by ^{15}N NMR spectrometry. Solutions of NANT (5–60 μM) (Fig. 1B) or of *N*-nitrosotryptophan residues in γ-globulin (0–10 μM) (Fig. 1C) in the presence of *N*-methyl-4-hydrazino-7-nitrobenzofurazan (MNBDH) (20 μM) were used in 2 mL phosphate buffer (50 mM, pH 7.4, 37 °C, 100 μM EDTA). The concentrations around the detection limit are additionally shown in the insert in Fig. 1C. After a reaction period of 2 h, ethanol (0.5 mL) was added to each sample and the formation of the fluorescence dye MNBDA was fluorimetrically determined at $\lambda_{em.}$ = 536 nm ($\lambda_{exc.}$ = 484 nm). The data shown are representative for three experiments performed independently (Fig. 1A) or are means of three experiments independently performed in duplicate (Fig. 1B and 1C).

of the MNBDH assay is given by the fact that the fluorescence of MNBDA is not quenched by proteins (Fig. 1C). The novel assay has a quantification limit of 250 nM nitrosotryptophan residues in the γ-globulin fraction (insert in Fig. 1C). This is the first example that *N*-nitrosotryptophan residues in proteins can be quantified by means of a relatively economical fluorescence method. Although *N*-nitrosotryptophan residues in other proteins may also be detected, the problem with the hydrolysis reaction still exists for the MNBDH assay, and the detection limit is much too high for a useful quantification in biological samples.

As noted above, stoichiometric amounts of nitric oxide are released from the reaction of Trolox with *N*-nitrosotryptophan derivatives.[41] The reaction proceeds with low molecular *N*-nitrosotryptophan compounds like NANT as well as with *N*-nitrosotryptophan residues in proteins. *S*-Nitrosothiols do not release any NO under such conditions because the experiments are performed in the presence of EDTA (which complexes the catalytically active transition metal ions required for decomposition of *S*-nitroso compounds). The influence of the always competing hydrolysis reaction can be reduced by working at pH 11 where the rate of the hydrolysis reaction is about 8-fold lower than at pH 7.4. Further, the rate of the reaction is increased at excess concentrations of Trolox. Since the Trolox-*N*-nitrosotryptophan reaction proceeds even under oxygen-free conditions, the liberated nitric oxide can be quantified by transporting nitric oxide into the gas phase where it may be detected with the very sensitive (5 pM) ozone-chemiluminescence method.[7] Considering all these facts, the Trolox assay is expected to reach a detection limit of about 5 nM for *N*-nitrosotryptophan compounds and is, therefore, recommended as a standard detection procedure.

Conclusions

N-Nitrosotryptophan derivatives can be both generated and denitrosated at physiological pH. Under such conditions, the denitrosation process starts with a so-called direct transnitrosation, *i.e.*, the NO^+ function is transferred from the *N*-nitrostryptophan derivative to various substrates without the formation of other intermediates, such as, *e.g.*, N_2O_3. Since the thus formed substrate-derived nitroso compound YNO often acts as a spontaneous nitric oxide releasing entity (for example, with catechols, serotonin, vitamin E, or vitamin C as substrates) or as a nitric oxide transfer agent (with, for instance, thiols as substrates), *N*-terminal blocked tryptophan derivatives feature all chemical capabilities necessary for action as an endogenous nitric oxide transporter. So far, the lack of a reliable detection procedure had prevented the accurate analysis of endogenous or applied *N*-nitrosotryptophan derivatives in biological samples. The methods suggested in this paper are believed to fill this gap.

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