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4-(*N*-Methylhydrazino)-7-nitro-2,1,3-benzooxadiazole (MNBDH): A Novel Fluorogenic Peroxidase Substrate**

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Enzymes are characterized by a range of attractive features for applications in analytical chemistry. Low analyte detection limits, even in complex matrices, are realized by their high catalytic activity and substrate selectivity. In this context, peroxidases are of special interest because of the possibility of coupling H_2O_2 detection with reactions yielding H_2O_2. In addition to the type and activity of the peroxidase, the lower detection limit of the enzymatic reaction is influenced by the chromogenic or fluorogenic substrate used, as the properties of the detected reaction product play a crucial role. A variety of organic compounds are used as chromogenic substrates, for example, aromatic amines like *o*-phenylenediamine (OPD),[1] 3,3′-5,5′-tetramethylbenzidine (TMB),[2] and also 2,2′-azinobis(3-ethylbenzothiazolin)-6-sulfonate as the diammonium salt (ABTS)[3]. In case of the more sensitive fluorogenic methods the *p*-hydroxyphenylcarboxylic acids, especially *p*-hydroxyphenylacetic acid (pHPA), have found widespread application.[4]

The main disadvantage of the *p*-hydroxyphenylcarboxylic acids and other fluorogenic substrates is the difference between the optimum pH for the enzymatic reaction, which lies in a moderately acidic range,[5] and for fluorescence detection of the products, where alkaline media are required.[4] A second drawback is the short wavelengths for the excitation maxima of the fluorophores. Furthermore, oxidation of the known fluorogenic substrates yields, in most cases, a mixture of reaction products rather than one well defined fluorophore[6] and, in some cases, not even the exact structure of the fluorescent compounds obtained could be elucidated.

Hydrazine reagents are the most popular group of derivatising reagents for carbonyl compounds.[7] In this work, we describe the use of a hydrazine reagent as a fluorogenic peroxidase substrate. Surprisingly, the nonfluorescent 4-(*N*-methylhydrazino)-7-nitro-2,1,3-benzooxadiazole (MNBDH), which was recently introduced as reagent for the determination of carbonyl groups[8] and nitrite ions,[9] is oxidized by H_2O_2 in presence of peroxidase (POD) to the intensively fluorescing 4-(*N*-methylamino)-7-nitro-2,1,3-benzooxadiazole (MNBDA) [Eq. (1)]. The identity of the reaction product was verified by NMR, UV/Vis, and fluorescence spectroscopy, mass spectrometry, and HPLC. The enzymatic reaction and fluorescence detection are carried out in a mildly

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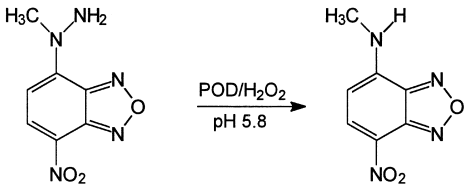

(1)

acidic medium (pH 5.8). In case of MNBDH, similar pH optima for conversion of the substrate and detection of the product are observed. The HPLC experiments, with UV/Vis detection of the reaction mixture, show only one additional peak in the chromatogram; its retention time and UV/Vis spectrum match that of pure MNBDA. With fluorescence detection (excitation wavelength: 470 nm, emission wavelength: 547 nm) only the MNBDA peak appears in the chromatogram. No other reaction products could be observed by liquid chromatography.

For the determination of H_2O_2, based on the procedure described below, the limit of detection (LOD) is 2.2×10^{-8} M and the limit of quantification (LOQ) is 7.5×10^{-8} M. These values are superior by a factor of three compared to those obtained for pHPA. In addition, the selectivity is improved using MNBDH because of the red-shifted excitation and emission maxima.

The MNBDH method was validated by comparison with two established peroxidase substrates, ABTS[10] and pHPA,[11] through the determination of glucose in four different beverages. Glucose is oxidized by aerial oxygen in the presence of glucose oxidase, and forms hydrogen peroxide. The latter is detected in a peroxidase-catalyzed reaction based on one of the substrates mentioned above.

The analytical figures of merit of the three methods are listed in Table 1. The photometric method had the smallest relative standard deviation but it is an order of magnitude less sensitive than the fluorimetric techniques. The LOD and LOQ with MNBDH are lower by a factor of three compared to pHPA. Further advantages of MNBDH are the red-shifted excitation and emission maxima (see also the fluorescence spectrum depicted in Figure 1) and an improved signal stability (Figure 2). Advantages for pHPA are the better water solubility and a higher reaction rate.

The results of the determination of α-D-glucose in soft drinks are presented in Table 2. Apart from the data for cola and lemon-flavored instant tea obtained by using pHPA, all results correspond well.

The MNBDH reagent may therefore be seen as prototype of a new and powerful class of peroxidase substrates for which directed optimization should yield substrates with increased potential.

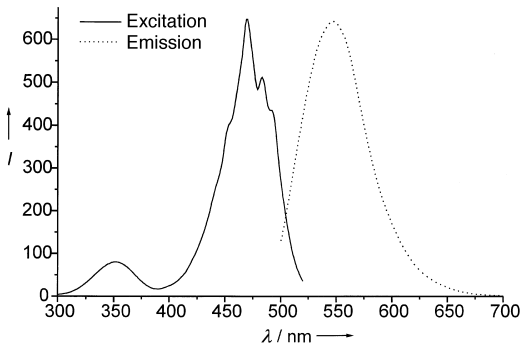

Figure 1. Fluorescence spectrum of MNBDA.

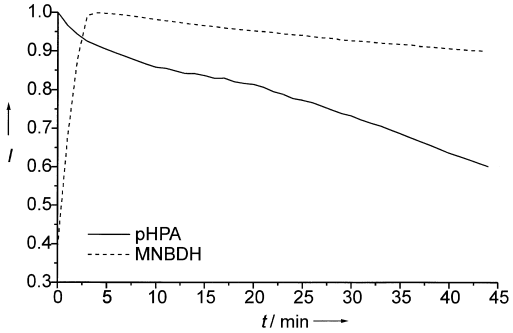

Figure 2. Time-based development for the reaction of MNBDH and pHPA with peroxidase and H_2O_2.

Table 2. Results for the determination of glucose in soft drinks. All values refer to the content of α-D-glucose.[a]

Method	Apple juice [g L^{-1}]	Orange juice [g L^{-1}]	Cola [g L^{-1}]	Instant tea [% w w^{-1}]
$ABTS_{405}$	20.6 ± 0.4	25.4 ± 1.1	41.1 ± 0.6	20.6 ± 1.0
$ABTS_{649}$	20.9 ± 0.6	26.0 ± 1.4	42.4 ± 0.8	20.6 ± 1.3
$ABTS_{732}$	21.0 ± 0.5	26.0 ± 1.0	42.7 ± 0.6	20.3 ± 1.0
MNBDH	22.0 ± 0.4	26.4 ± 0.5	44.2 ± 2.1	20.0 ± 1.2
pHPA	20.8 ± 2.1	26.1 ± 1.4	50.0 ± 1.7	15.3 ± 1.4

[a] Deviations are calculated as the standard deviation of the mean value of eight separate determinations.

Experimental Section

Commercial samples investigated in this study were apple juice, orange juice, cola, and lemon-flavored instant tea. Liquid samples were diluted directly (by a factor of 10000 for detection with ABTS or pHPA, and by a

Table 1. Parameters for the determination of glucose using the peroxidase substrates ABTS, MNBDH, and pHPA.

Method	Limit of detection [M][a]	Limit of quantification [M][b]	Averaged standard deviation [%][c]	Smallest/largest standard deviation [%][d]	Linear range [M]
$ABTS_{405}$[e]	6×10^{-7}	2×10^{-6}	2.7	5.7/0.3	$2 \times 10^{-6} - 2 \times 10^{-4}$
$ABTS_{649}$	1×10^{-6}	3×10^{-6}	2.8	7.3/0.4	$3 \times 10^{-6} - 2 \times 10^{-4}$
$ABTS_{732}$	5×10^{-7}	2×10^{-6}	1.9	4.2/0.4	$2 \times 10^{-6} - 2 \times 10^{-4}$
MNBDH	5×10^{-8}	2×10^{-7}	3.6	5.6/2.7	$2 \times 10^{-7} - 2 \times 10^{-5}$
pHPA	2×10^{-7}	5×10^{-7}	5.3	8.8/2.7	$5 \times 10^{-7} - 2 \times 10^{-5}$

[a] Calculated as the triple standard deviation of the blank. [b] Calculated as the tenfold standard deviation of the blank. [c] Relative standard deviation of eight separate determinations, averaged for the linear range. [d] Relative standard deviation of eight separate determinations, smallest and largest value of the linear range. [e] Subscripts correspond to the wavelengths (in nanometers) used for photometric measurements.

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factor of 50000 for MNBDH). The instant tea was weighed and dissolved in bidistilled water (0.5 g in 10 mL water) and diluted in the same way.

All measurements were carried out using microplate readers.

Glucose oxidase (GOD) reaction (I): 50 μL of a solution of GOD (15 mg) in acetate buffer (10 mL; pH = 5.5; 0.01M) were added to 100 μL of a glucose solution. After mixing thoroughly, the solution was incubated for 15 min at 37 °C.

GOD reaction (II): as I, but 20 μL GOD solution and 40 μL glucose solution were used.

GOD reaction (III): as I, but the GOD solution was prepared with phosphate buffer (pH 5.8; 0.01M).

Glucose determination using POD and ABTS: 50 μL of a solution containing POD (0.5 mg) and ABTS (5.5 mg) in acetate buffer (10 mL; pH 5.5; 0.01M) were pipetted to the mixture of reaction I. After the sample had been mixed thoroughly and incubated for 10 min at room temperature, the absorbance of the samples was measured at 405, 649, and 732 nm.

Glucose determination using POD and pHPA: 50 μL of a solution containing POD (2.5 mg) and pHPA (7.6 mg) in ammonium buffer (10 mL; pH 9.5; 0.01M) were pipetted to the mixture of reaction II. After the sample had been mixed thoroughly and incubated for 15 min at room temperature, the fluorescence of the samples was measured at excitation and emission wavelengths of 320 and 405 nm, respectively.

Glucose determination using POD and MNBDH: 1 mg MNBDH was dissolved in 10 mL acetonitrile. Of this solution, 1.4 mL are added to a solution containing POD (2.5 mg) in phosphate buffer (10 mL; pH 5.8; 0.01M). From this mixture, 40 μL were pipetted into the mixture of reaction III. After the sample had been mixed thoroughly and incubated for 10 min at room temperature, the fluorescence of the samples was measured at excitation and emission wavelengths of 470 and 545 nm, respectively.

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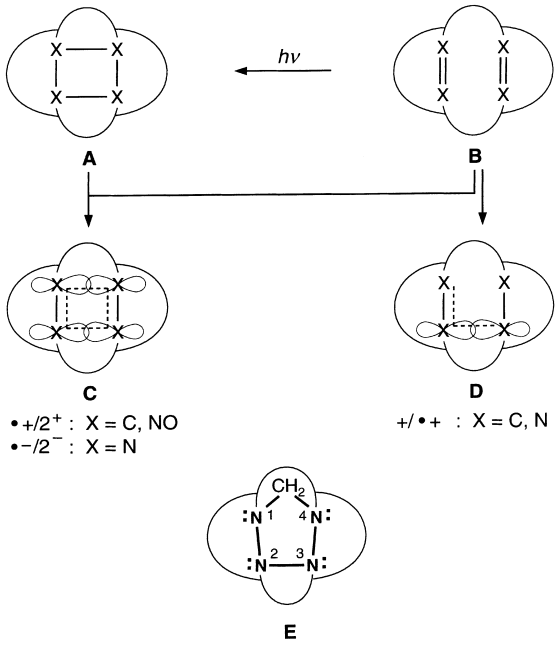
[10] ABTS is oxidized to an intensely green radical cation and shows UV/Vis absorption maxima at 405, 415, 649, 732, and 815 nm.

[11] The phenol derivative pHPA is dimerized in the peroxidase-catalyzed reaction to form the corresponding biphenol, which fluoresces in alkaline media (pH 9.5) with excitation and emission maxima of 323 and 403 nm, respectively.

σ-Homoconjugation in Cyclically Preoriented N4-(Radical) Cations—N ··· N Bond Lengths > 2 Å**

Kai Exner,* Birgit Großmann, Georg Gescheidt, Jürgen Heinze, Manfred Keller, Thomas Bally, Pavel Bednarek, and Horst Prinzbach*

(pp)σ-Conjugation, the electron delocalization between collinear p-orbitals, is a borderline case for the theory of chemical bonding.[1] It occurs efficiently only in compounds difficult to synthesize and with specific steric/stereoelectronic prerequisites. Making use of cage-fixed cyclobutanes **A** (X = C, (iso)pagodanes), of preoriented dienes **B** (X = C, (iso)-pagodadienes/(seco)dodecahedradienes), and bisdiazenes/bisdiazenetetroxides **B** (X = N, NO), the effective σ-homoconjugation or σ-bishomoaromaticity in 3C/3(2)e cations **D** (X = C),[2] in 4C(N)/3(2)e cations **C** (X = C, NO),[3, 4] and in 4N/5(6)e anions **C** (X = N)[5] have been observed. Here, we



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