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Peroxidase catalyzed formation of azine pigments—a convenient and sensitive method for the identification of human cells with positive myeloperoxidase reactivity

Anja Bodtke,[a] Wolf-Diethard Pfeiffer,[a,*] Norbert Ahrens[b] and Peter Langer[a,*]

[a]*Institut für Chemie und Biochemie der Ernst-Moritz-Arndt-Universität Greifswald, Soldmannstr. 16, D-17487 Greifswald, Germany*
[b]*Klinik für Neurologie der Ernst-Moritz-Arndt-Universität Greifswald, Ellernholzstr. 1/2, D-17487 Greifswald, Germany*

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Abstract—A new and efficient method for the identification of human cells with positive myeloperoxidase reactivity was developed. This method is based on the myeloperoxidase catalyzed formation of *p*-naphthoquinone-thiazol-2-on-azine pigments and offers a number of advantages compared to standard methods. In addition, we report the horseradish peroxidase (HRP) catalyzed oxidative coupling of 2-hydrazono-4-thiazolines with α-naphthol. This transformation allows a new and efficient synthesis of *p*-naphthoquinone-thiazol-2-on-azines under mild conditions.
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The exact diagnosis of inflammatory processes and malignant diseases of white blood cells (e.g., leukemia) and of diseases with a change of age steps of neutrophilic granulocytes is of high importance for an efficient therapy.[1] The age of neutrophilic granulocytes can be studied by detection of the catalytic activity of myeloperoxidase.[2] In addition, myeloperoxidase reactivity represents a probe for the identification of histologic structures of many organs. Therefore, efficient and sensitive methods for the identification of cells with positive myeloperoxidase reaction are of great importance for clinical chemists.

The standard method used for more than 30 years, based on work of Graham and Knoll[3] and Graham and Karnovsky,[4] relies on the myeloperoxidase catalyzed oxidation of *O*-toluidine, diaminobenzidine or (less toxic) polyalkylbenzidines with hydrogen peroxide. In these reactions, blue product mixtures are formed which mainly contain quinones and semiquinones.[5–8] The mixtures slowly undergo a rearrangement into yellow-brownish pigments of low water solubility. In combination with the application of osmium(VIII) tetroxide the method of Graham generally gives good results for electron microscopical analyses of the dyed cytologic material. However, there are several drawbacks. Firstly, a direct *light* microscopic analysis is not possible, due to the pale yellow-brownish colour of the pigment. Therefore, the use of a contrast medium (alaun) is necessary; the sample preparation requires additional steps and is not user-friendly. Secondly, a reproducible localization of the peroxidase containing cell districts by light microscopic inspection of the cytologic material is difficult. A major disadvantage lies in the fact that the judgment of the enzymatic activity close to the cell nucleus is not possible. However, the myeloperoxidase activity in neutrophilic granulocytes mainly occurs close to the cell nucleus. Thirdly, the chemical structure of the pigments is not defined and, dependent on the conditions (pH), mixtures of varying composition are formed. Other problems are the light sensitivity of the products and the occurrence of follow-up reactions. The methodology reported herein (a) does not require the use of a contrast medium, (b) allows a judgment of the enzymatic activity close to the cell nucleus and (c) a well-defined pigment is formed.

Herein, we wish to report a convenient and sensitive method for the identification of cells with positive myeloperoxidase reaction. Our methodology relies on the myeloperoxidase catalyzed reaction of 3-alkyl-2-hydrazono-4-thiazolines with α-naphthol in the presence of hydrogen peroxide to give violet *p*-naphthoquinone-thiazol-2-on-azines.[9] The microscopical grading allows

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* Corresponding author. Tel.: +49-3834-864461; fax: +49-3834-864373; e-mail: peter.langer@uni-greifswald.de

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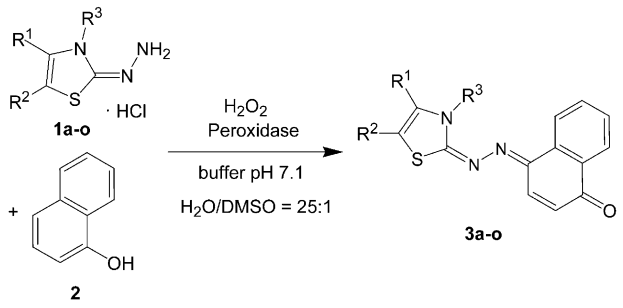
a precise differentiation of enzyme activity. Therefore, the method is of considerable importance for the diagnosis of inflammatory processes and malignant diseases of white blood cells (e.g., leukemia) and diseases with a change of age steps of neutrophilic granulocytes. As a test system, we first studied the horseradish peroxidase (HRP) catalyzed oxidative coupling of 3-alkyl-2-hydrazono-4-thiazolines with α-naphthol in the presence of hydrogen peroxide. This transformation not only represents a model for the myeloperoxidase catalyzed azine formation in blood cells, but can be also used for the efficient synthesis of azines under mild conditions.[10] Horseradish peroxidase represents a haem-containing enzyme.[10,11] The catalytic pathway of horseradish peroxidase (HRP) has been recently studied by X-ray structure analysis.[11]

The potassium hexacyanoferrate(III) mediated reaction of hydrazone **1a**, available in a few synthetic steps based on known methods,[12] with α-naphthol (**2**) afforded the *p*-naphthoquinone-thiazol-2-on-azine **3a**—a meroyanine dye containing a *push–pull* heterocyclic system—in quantitative yield.[13] The hydrogen peroxide mediated, HRP catalyzed reaction of **1a** with **2** was next studied. The addition of horseradish peroxidase resulted in the formation of **3a** which was isolated, after thorough optimization of the conditions, in up to 99% yield.[14] In the absence of peroxidase the rate of the reaction was extremely low and almost no conversion was observed. The starting material **1a** had to be used in the form of the hydrochloride, due to its optimal water solubility.

To study the preparative scope of the transformation and to optimize the physical properties of the azine a number of different 2-hydrazono-4-thiazolines **1** were prepared and their peroxidase catalyzed condensation with α-naphthol was studied (Scheme 1, Table 1). The reaction of **2** with thiazolines **1a**–**o** afforded, on a 1 mmol scale, the *p*-naphthoquinone-thiazol-2-on-azines **3a**–**o** in very good yields. The products were obtained as green to nearly black crystals; the colour of the solution varied in the range from red to deep violet.

A number of cell experiments were successfully carried out. We have found that the formation of azine **3a** was catalyzed by myeloperoxidase present in human blood or peroxidase rich material (e.g., pus).[15] The histo-cytologic material was fixed by vapour-phase contact with formalin (35%). This method is milder than the use of methanol; it has been noted that the latter possesses peroxidase damaging properties. Incubation of the material with a solution of **1a**, **2** and H_2O_2 (see Experimental Section) resulted in a positive peroxidase reaction. The extension of the incubation time from 5 to 25 min did not result in any improvement. After many trial experimentations we have been able to minimize the amount of organic solvent (DMSO or DMF), necessary to solubilize α-naphthol, from 9% to 1.1%. This optimization was extremely important since organic solvents are known to damage the cell membrane which results in a leakage of the pigment.

Inspection of the plasma by microscopy (Fig. 1) revealed a grading (from yellow to deep red) which allowed a precise differentiation of the enzyme activity within the cell and of the age of neutrophilic granulocytes. These diagnostic information are very important for an optimal therapy of myeloic leukoses. Direct microscopical analysis of the cytologic material showed that most of the pigment **3a** was formed close to the cell nucleus. Due to the excellent visibility of pigment **3a**, the sample could be analyzed directly by microscopy.



Scheme 1. Peroxidase catalyzed synthesis of pigments **3a**–**o**.

Table 1. Products and yields

3	R^1	R^2	R^3	Appearance[a]	mp (°C)	(%)[b]
a	Ph	Me	Me	Green prisms (B)	275	99
b	Ph	Me	*i*Pr	Red-brown lamella (B)	157–158	96
c	Ph	Me	Ph	Violet lamella (B)	244	96
d	Ph	Et	Ph	Black-green lamella (B)	205	95
e	Me	Ph	Ph	Black-violet lamella (C)	208	99
f	Ph	Ph	Me	Deep red needles (D)	197	99
g	Ph	Ph	Ph	Black-green prisms (A)	203	99
h	Ph	Et	Me	Deep red needles (E)	188	99
i	Me	Ph	Me	Red-brown needles (F)	205	98
j	Me	CO_2Et	Me	Red-brown needles (B)	261–263	90
k	Ph	Ph	Allyl	Deep green prisms (B)	194–195	90
l	Ph	Ph	*i*Pr	Deep green prisms (B)	229–230	92
m	Ph	Ph	*n*Bu	Deep green lamella (B)	212	98
n	Ph	Ph	*i*Bu	Deep green prisms (B)	229–230	91
o	Me	Ph	NH_2	Red-brown needles (G)	237	99

[a] In brackets: crystallization solvent: A: *n*PrOH, B: $EtOCH_2CH_2OH$, C: C_6H_6, D: dioxane/EtOH, E: $EtOCH_2CH_2OH$/EtOH, F: dioxane, G: EtOAc.
[b] Yields of isolated products.

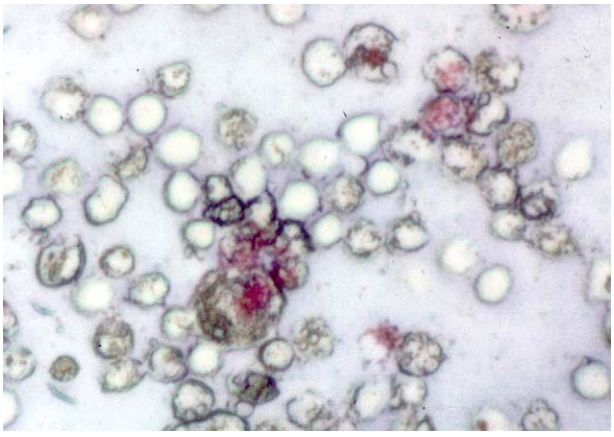


Figure 1. Microscopic picture of cells (granulocytes) with positive peroxidase reaction and phagocytosis of a granulocyte by a macrophage.

The use of a contrast medium was not required. This simplifies the sample preparation and guarantees a better differentiation of the enzyme activity by microscopy.

To optimize the solubility of **1** and **3** and the optical features of **3** the substituents of the starting materials **1** were systematically varied. The best results were obtained for azine **3a**. Hydrazone **1b** and the corresponding azine **3b** gave good results in terms of solubility; however, the microscopic inspection of the incubated cells revealed that the red colour of the reactive cells was too pale. Other derivatives **3** proved unsatisfactory in terms of solubility and/or optical features (Table 1). The presence of specific substituents (R^1 = Ph; R^2 = Ph, Alkyl; R^3 = Ph, Alkyl) proved mandatory to guarantee a sufficient stability of reagent **1** in aqueous solution. An optimal water solubility of hydrazones **1** was observed for R^2 = Me. In contrast, the presence of an aryl group gave disappointing results. The best optical results were obtained for derivatives containing substituents R^1 = Ph and R^3 = Me.

Similar to the established methods, the reaction reported herein should be specific for the myeloperoxidase mediated oxidative system ($H_2O_2 + Cl^- + H^+ \rightarrow HOCl + H_2O$). Oxygen dependent intracellular oxidative systems,[16] such as reactions of NADPH-oxidase ($NADPH + 2O_2 \rightarrow 2O_2^- + NADP^+ + H^+$) or NO-synthease (2 L-Arginine $+ 4O_2 + 3\,NADPH + 3H^+ \rightarrow 2$ L-Citrulline $+ 2\,NO^\bullet + 4H_2O + 3\,NADP^+$), are irreversibly blocked by the use of formalin during the fixation (the radicals are quenched by reaction with formalin). The H_2O_2 dependent glutathioneperoxidase mediated oxidative system [H_2O_2 + Glutathione (red.)$\rightarrow 2H_2O$ + Glutathione (ox.)] should not be problematic, due to the use of an excess of hydrogen peroxide. An influence of katalase activity ($2H_2O_2 \rightarrow 2H_2O + O_2$) can not be excluded. However, the catalase activity was low and inverse to the concentration of H_2O_2.[2] Ten control experiments were carried out using material containing only monocytes and lymphocytes which are known to contain only small quantities of myeloperoxidase. The cytologic material was prepared from 1 mL of cerebrospinal fluid (CSF) by cell enrichment (sedimentation) according to J. Sayk.[17] Negative results were obtained using our test system.

Acknowledgements

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14. *Synthesis of [1,2-diaza-2-(3,5-dimethyl-4-phenyl-1,3-thiazolin-2-ylidene)ethylidene] naphthal-en-1-one* (**3a**). To an aqueous solution (100 mL) of hydrazone **1a** (255 mg, 1 mmol) was added a solution (H_2O/DMSO = 3.5:1 or H_2O/DMF = 3.5:1, 90 mL) of α-naphthol (145 mg, 1 mmol). To the mixture was added dropwise an aqueous solution of H_2O_2 (0.15 mL, 30%). Addition of an aqueous solution (sodium phosphate buffer pH 7.1, 10 mL, 0.1 M) of horseradish peroxidase (10 mg, specific activity 533 mmol/s·kg protein) resulted in the formation of a precipitate. After addition of H_2O, the precipitate was filtered off, washed with H_2O and recrystallized from 2-ethoxyethanol to give **3a** as green prisms (0.72 g, 99%), mp 275 °C. ^{1}H NMR ($CDCl_3$, 300 MHz). δ = 1.61 (s, 3H, Me), 2.12 (s, 3H, Me), 6.60–8.51 (m, 10H, ArH, Ar). ^{13}C NMR ($CDCl_3$, 75 MHz): δ = 12.80, 34.21, 114.73, 123.01, 125.90, 128.03, 128.10, 128.65, 129.08, 129.28, 129.56, 130.23, 130.42, 131.74, 135.73, 136.22, 143.72, 172.76, 186.15. IR (KBr, cm^{-1}): $\tilde{\nu}$ 769 (m), 825 (m), 1002 (m), 1130 (s), 1241 (m), 1309 (s), 1388 (s), 1410 (s), 1438 (s), 1473 (s), 1536 (s), 1592 (s), 1630 (s), 2061 (s). UV (EtOH, nm): λ_{max} (lgε) = 244 (4.41), 300 (4.27), 530 (4.66). Anal.: calcd for $C_{21}H_{17}N_3OS$ (359.4): C 70.18, H 4.77, N 11.69. Found: C 69.90, H 5.16, N 11.88.
15. *Cell experiments:* The following solutions, which are storable in the refrigerator at 0 °C, were prepared: *Solution 1*: Thiazoline **1a** (255 mg, 1 mmol) was dissolved in water (100 mL). *Solution 2*: To a DMSO solution (2.5 mL) of α-naphthol (145 mg, 1 mmol) was added water (97.5 mL) and an aqueous solution of phosphate buffer (pH 7.1, 10 mL, 0.1 M). *Solution 3*: An aqueous solution of H_2O_2 (30%). To *solution 2* (10 mL) were added *solution 3* (15 μL) and *solution 1* (10 mL) to give *solution 4*. The histo-cytologic material (30–50 μL) was fixed for 1–3 min by vapour-phase contact with formalin (35%), washed with water and dried in the air. Incubation of this material with *solution 4* (1 mL) for 5–10 min and subsequent washing with water resulted in a positive peroxidase reaction which could be directly observed by microscopy (see Fig. 1). A quantification of the enzyme activity was not carried out.
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