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Synthesis and peroxidase-staining properties of novel water soluble polyhydroxylalkyl benzidine dyes

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Abstract

A series of water soluble *N*-polyhydroxyalkyl benzidine derivatives was synthesized. The oxidation of glucose–benzidine and fructose–benzidine by iodide was faster than that of benzidine, while *N*-substituted hydroxylethyl and *N*-mercaptoethyl benzidines were almost the same as benzidine. It was found that these compounds were able to stain peroxidase well, and compound **2** (fructose–benzidine conjugate) gave the best visualization, which was as good as benzidine.

Keywords: Benzidine; Glucose; Fructose; Hydroxylethyl; Mercaptoethyl; Peroxidase; Oxidation; Staining; Leukemia

1. Introduction

Leukemia is distinguished through staining by fixing the amount of peroxidase inside a white cell. Benzidine is often used for this purpose clinically. In the presence of the peroxidase, benzidine could be converted into oxidized benzidine by fresh oxygen released from hydroperoxide. The complex, which was formed by oxidized benzidine binding with benzidine, is combined with sodium nitroferricyanide inside the cell to initially give a blue color which turns brownish-black after further oxidization by peroxidase. The diagnosis of leukemia is based on the extent of the staining color of the peroxidase.

Benzidine is a well-known chemical and chromophore, which has been widely used. As dyes and pigments derived from benzidine can cause serious health problems for humans, benzidine is almost no longer used in the synthetic dyestuff industry for textiles, and research has focussed on finding a substitute for benzidine. Usually, the modification of benzidine by the introduction of certain moieties, especially water-soluble groups, such as $-SO_3H$, can decrease its toxicity and prevent its mutation.

In fact, benzidine still plays an important role in cell biology and clinical work as a staining agent [1,2]. Although the amounts of benzidine dyes used for biological purposes were very small and not comparable to the amount used in the textile industry, benzidine can potentially cause cancer and mutation and therefore, the research to find novel peroxidase-staining agents to replace benzidine has attracted a lot of attention.

For staining applications in cell biology and the diagnosis of leukemia, several novel benzidine derivatives with high oxidation potentials have

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been synthesized and tested [3–6]. Electron-donating groups, e.g. methyl, methoxyl, hydroxyl, and phenyl, were introduced to the phenyl moiety of benzidine. In addition, methyl and phenyl groups were also used to modify the amino moiety of benzidine. Here, we report the synthesis and peroxidase-staining properties of novel substituted benzidine derivatives, e.g., mercapto-, *N*-(β-hydroxyethyl)-, *N*-(β-mercaptoethyl)- group to the amino-moiety of benzidine We hoped to find highly efficient peroxidase-staining agents with low toxicity and good water-solubility (Scheme 1).

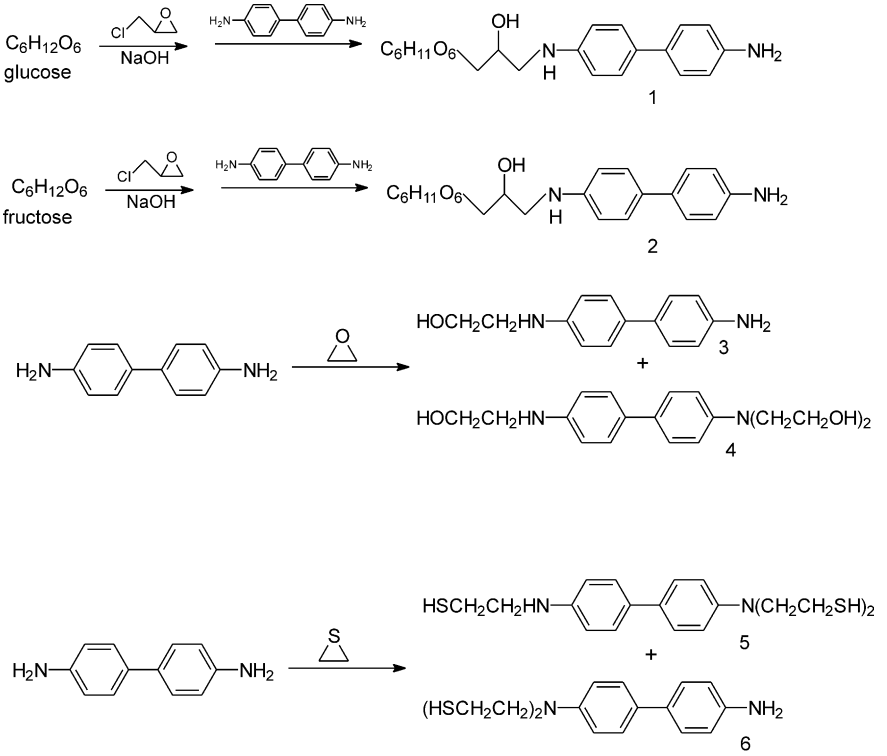
2. Results and discussion

2.1. Synthesis

First, in order to decrease the toxicity of the benzidine, polyhydroxyl groups were introduced through a alkylene epoxide linker, since polyhydroxyl compounds are usually water-soluble and less toxic. The resulting polyhydroxyalkyl–benzidines might bind with cells more easily. The linker used here was ethylene epoxide, which is readily available and has been used as a linker between the amino groups of proteins and the solid support in the chromatographical purification of protein-enzymes. Secondly, in order to study the effect of linkers on staining we also prepared some *N*- hydroxy- and mercapto- ethyl benzidines.

Glucose was condensed with chloropropylene epoxide in an aqueous sodium hydroxide solution, prior to mixibng with benzidine to give a conjugate of glucose–benzidine (**1**). The fructose–benzidine conjugate (**2**) was prepared in a similar way. They all showed absorption in the 3100–3600 cm^{-1} range for hydroxyl or amine groups in IR, and gave mass peak at 439 ($M+1+H_2O$) and 421 ($M+1$), respectively.

Benzidine reacted with ethylene epoxide in a sealed tube to give mono-substituted *N*-(β-hydroxylethyl)benzidine and tri-substituted *N,N,N′*-tris(β-hydroxylethyl)benzidine in the ratio of



Scheme 1. Synthetic route of benzidine derivatives.

about 1:2. Their structures were confirmed by using IR, MS and ^{1}H NMR. Benzidine and ethylene sulfide were refluxed in benzene to afford disubstituted *N,N*-(β-mercaptoethyl)benzidine (**6**) and tri-substituted *N,N,N′*-(*β*-mercaptoethyl)benzidine (**5**) in a ratio of approximate 1:3. Their structures were also confirmed by using IR, ^{1}H NMR and MS. Compound **6** showed four doublet peaks in the aromatic proton area in ^{1}H NMR which implied that it was not a *N,N′*-disubstituted product, rather *N,N*-disubstituted.

2.2. Staining properties

2.2.1. Comparison of oxidation of compounds with iodide

The staining of benzidine by peroxidase was an irreversible oxidation process (*to* produce a black color). From the observation on the silica gel plate it was found that the oxidation of glucose–benzidine and fructose–benzidine by iodide was faster than of benzidine alone; the oxidation of *N*-substituted hydroxyethyl or mercaptoethyl benzidine proceeded at almost the same rate as benzidine.

2.2.2. The staining properties of compounds

Compounds **1**, **2** and **4** were used for the staining of leukemia cells inside the bones of patients, and compared with that of benzidine. The staining images were obtained under microscopy at 20 and 40 multiples, respectively. The pictures as shown in Fig. 1.

It was found that these three compounds were able to stain peroxidase inside leukemia cells in a similar way to benzidine. Compound **2** (fructose-benzidine conjugate) gave the best visualization, which was as good as benzidine alone. Compound **2** was worth studying further as a novel and water-soluble staining agent for peroxidase

3. Experimental

3.1. Synthesis

3.1.1. Synthesis of glucose–benzidine conjugates

3.1.1.1. Activation of glucose. Glucose (12.0 g, 66.7 mmol), sodium hydroxide solution (4.80 g,

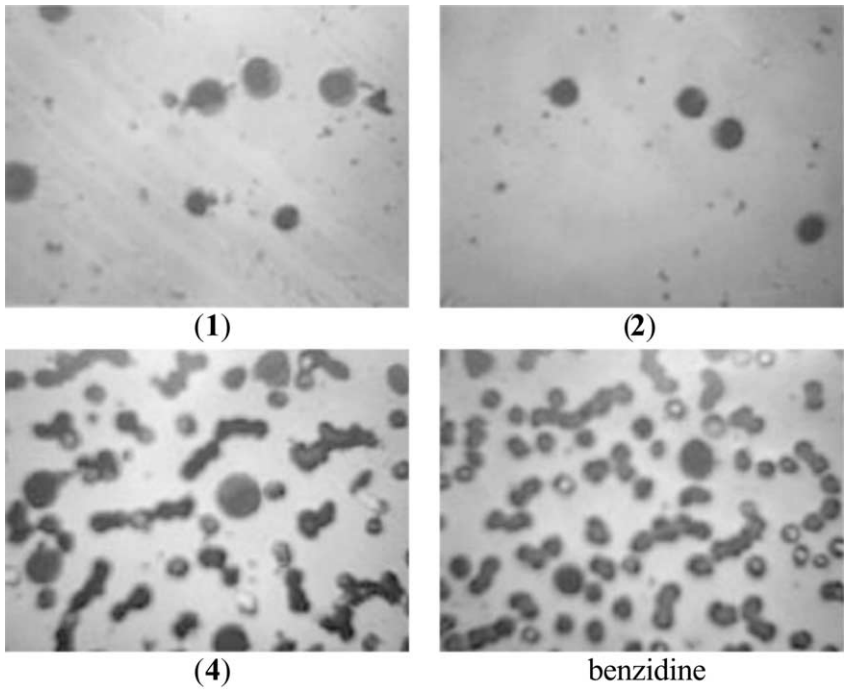


Fig. 1. The photographs of staining of leukemia cells (×40) for compounds **1**, **2** and **4**.

water 60 ml), and 1,4-dioxohexane solution (50 ml, water 60 ml) were mixed at room temperature. Chloropropyl epoxide (4.8 ml, 60 mmol) was added dropwisely to the above mixture. The reaction was stirred at 40–45 °C for 4 h to give a clear colorless solution.

3.1.1.2. Coupling. Benzidine (11.0 g, 60.0 mmol) was added to the above mixture for 10 min. The mixture was stirred for 5 h at 40–45 °C until the benzidine dissolved. After removing the dark residue on the bottom of the flask, the clear solution was evaporated to give a reddish solid (13.4 g). The rude product was crystallized from 95% alcohol (20 ml) at −20 °C to give a colorless solid (4.40 g, 17.4%): IR (KBr, cm^{-1}) 3500–3100 (*s*, OH, NH), 2900 (CH), 1610,1510 (C=C), 1020–1070, 810 (*p*-disubstituted benzene CH); ESI-MS, *m*/*z* (relative intensity) 439 (94.2, M + 1 + H_2O), 421(20, M + 1), 301(94.8), 277(18.7, M + H_2O-$C_6H_{11}O_6$), 259 (23.35, M-$C_6H_{11}O_6$), 203 (100), 85 (83.8), 55 (48.3).

3.1.2. Synthesis of fructose-benzidine conjugates

3.1.2.1. Activation of fructose. Similar to the in Section 3.1.1.1. procedure, fructose (10.40 g, 57.6 mmol) was used instead of glucose.

3.1.2.2. Coupling. Similar to the procedure in Section 3.1.1.2. , benzidine (10.50 g, 57.0 mmol) was added to the mixture. The crude product (9.7 g) was recrystallized from alcohol at −20 °C to give a colorless solid (5.80 g, 24.1%): IR (film, cm^{-1}) 3100–3600 (*br*, OH, NH), 2950 (CH), 1600, 1510 (C=C), 1420 (*br*,), 1250 (*br*,), 810 (*p*-disubstituted benzene CH), 600 (*br*,); ESI-MS, m/z (relative intensity) 439 (27.1, M + 1 + H_2O), 421 (6.5, M + 1), 383 (33.3, M + H_2O-C_3H_4O), 301 (58.1), 277 (18.7, M + H_2O-$C_6H_{11}O_6$), 259 (51.6, M-$C_6H_{11}O_6$), 203 (100).

3.1.3. Synthesis of N-*hydroxylethylbenzidine derivatives (**3** and **4**)*

Ethylene epoxide, a syringe, a stainless sealed tube (25 ml) and toluene were cooled in a refrigerator for 0.5 h. Benzidine (3.32 g, 18.02 mmol), ethylene epoxide (6.6 ml) and toluene (20 ml) were mixed in the sealed tube. After stirring at 80–90 °C for 15 h, the reaction was cooled in ice water for 30 min and filtrated. The crude solid (3.17 g) was purified by column chromatography (silica gel, petroleum ethe: EtOAc/1:2) to give the product **3** (0.70 g, 17.1%) and **4** (1.88 g, 38.4%), respectively.

3.1.3.1. N-*(β-hydroxylethyl)benzidine* (***3***). R_f = 0.40 (petrolum ether/EtOAc, 1:2,); mp 117–118 °C; IR (KBr, cm^{-1}) 3400 (*br*, OH, NH), 3050 (*w* CH), 2920, 2850 (CH), 1610, 1510, 1450 (C=C), 1280, 1050, 810 (CH, *p*-disubstituted benzene), 510; EI-MS, m/z (relative intensity) 228 (95.7, M +), 197 (100, M-$HOCH_2$), 184 (58.6, M-CH_2CH_2O), 167 (17.0, M-$C_6H_4C_6H_4$), 149 (52.6); ^{1}H NMR(DMSO-d_6, 100MHz) δ 7.23 (*m*, 4H), 6.58 (*d*, *J* = 6.0 Hz, 4H), 5.45 (*br*, 2H, OH and NH), 4.76 (*t*, *J* = 5.25 Hz, 1H), 4.72 (*t*, 1H), 3.54 (*t*, *J* = 7.35 Hz, 2H), 3.08 (*t*, *J* = 5.5 Hz, 2H).

3.1.3.2. N,N,N′-*tris (β-hydroxylethyl)benzidine* (***4***). R_f = 0.20 (petroluem ether/acetate, 1:2); mp 143–144 °C; IR (KBr, cm^{-1}) 3500–3200 (*br*,), 3050 (ArH), 2950, 2850, 1610, 1510, 1450 (C=C), 1380, 1180, 1060, 810 (s, *p*-disubstituted benzene), 510; EI-MS m/z (relative intensity) 316 (14.2, M +), 284 (26.6), 229 (9.6), 197 (26), 196 (24), 105 (11.3), 44 (14.8); ^{1}H NMR (DMSO-d_6, 100 MHz) δ 7.33 (*d*, *J* = 8.74 Hz, 2H), 7.28 (*d*, *J* = 8.47 Hz, 2H), 7.28 (*d*, *J* = 8.47 Hz, 2H), 6.71 (*d*, *J* = 8.8 Hz, 2H), 6.62 (*dd*, *J* = 1.8, 8.6 Hz, 2H), 5.43 (*br*, s, 1H), 4.75 (*br*, s, 2H), 3.56 (*t*, *J* = 5.0 Hz, 4H), 3.41 (*t*, *J* = 6.4 Hz, 2H), 3.11 (q, *J* = 5.5 Hz, 2H).

3.1.4. Synthesis of N-*(mercaptoethyl)benzidine derivatives (**5** and **6**)*

To an aqueous KSCN solution (160 g, 1.6464 mol, 300 mL water) at −10 °C, refrigerated ethylene epoxide (30 ml) was added. After stirring at −10 °C for 2 h and standing for over 6 h, water was removed using a separation funnel and the resulting emulsion was filtered. The filtrate was dried over $CaCl_2$ to give oily liquid. Ethylene sulfide (8 ml) was collected by distallation at 52–56 °C. This distillate and benzidine (2.1 g, 11.40 mmol) in benzene (100 ml) was refluxed for 24 h. Evaporation of the solvent yielded a reddish-yellow solid (6.25 g mmol). A portion of the crude product (0.25g) was purified by column

chromatography (silica gel, petroleum ether:-EtOAc/2:1) to give **5** (0.019 g, 11.5%) and **6** (0.052 g, 37.8%), respectively.

3.1.4.1. N,N,N′-*tri*(β-*mercaptoethyl*)*benzidine* (***5***). $R_f = 0.75$ (petroleum ether:ethyl acetate/2:1); mp 130–132 °C; IR (film, cm^{-1}) 3490 (NH), 3050, 2920, 1610, 1510, 1450 (C=C), 1350, 1180, 810 (CH). EI-MS, *m*/*z* (relative intensity) 362 (6.73, M+), 302 (8.4, M-C_2H_4S), 256 (6.5%, M-SCH_2), 152 (7.9, 2 C_6H_4), 60 (10.0, C_2H_4S); ^1H NMR ($CDCl_3$, 100 MHz) δ 7.53(*m*, 4H), 6.68 (*dd*, $J = 7.9$, 2.0 Hz, 4H), 3.98 (*t*, 5.5 Hz, 6H), 3.15 (*t*, 5.4 Hz, 6H), 2.80 (*br*, s, H).

3.1.4.2. N,N′-*di*(β-*mercaptothyl*)*benzidine* (***6***). mp 126–127 °C; $R_f = 0.37$ (petroleum ether:-EtOAc/2:1); IR (film, cm^{-1}) 3300–3600 (NH), 3030 (CH), 2920, 2880, 2550 (SH), 1610, 1510, 1450, 1420 (C=C), 1270, 1200, 1050, 810 (CH), 700, 510; EI-MS *m*/*z*, (relative intensity) 304 (12.7, M+), 302 (47.8), 244 (26.7, M-C_2H_4S), 197 (52.9), 151 (10.8), 121 (26.5), 105 (100); ^1H NMR ($CDCl_3$, 100MHz) δ 7.42 (*d*, $J = 6.7$ Hz, 2H), 7.36 (*d*, $J = 6.8$ Hz, 2H), 6.77 (*d*, $J = 8.3$ Hz, 2H), 3.70 (*m*, 4H), 2.92 (*br*, s, H), 2.75 (*m*, 4H).

3.2. Staining properties

The cell slurry concentrated from 1 ml of bone marrow was spread on a film. One drop of staining solution was added to the film. The film was allowed to stand for 1 min, then one drop of NaOH solution (1%) was added to the film and the film was allowed to stand for 4 min. After washing with water and standard washing solutions and drying, the staining photo-pictures were taken using microscopy with 20 and 40 multiples.

The staining solutions were composed of as following: (1) benzidine 0.3g, 95% alcohol 99 ml, sodium nitroferricyanide 360 mg; (2) compound **1** 1.2 g, deionized water 40 mg, sodium nitroferricyanide 509 mg; (3) compound **2** 4.747 g, 95% alcohol 60 mg, sodium nitroferricyanide 497 mg; (4) compound **4** 0.188 g, 95% alcohol 40 mg, sodium nitroferricyanide 198 mg.

4. Conclusions

Water-soluble *N*-polyhydroxylalkyl benzidine derivatives, e.g. glucose–benzidine, fructose–benzidine, *N*-hydroxylethylbenzidines and *N*-mercaptothylbenzidines, have been easily synthesized. The oxidation of glucose–benzidine and fructose–benzidine by iodide was faster than that of benzidine alone, while the oxidation of *N*-substituted hydroxyethyl and mercaptoethyl benzidines were almost same as benzidine. It was found that compounds **1**, **2**, and **4** were able to stain peroxidase well, and compound **2** (fructose-benzidine conjugate) gave the best visualization, which was as good as benzidine.

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