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Synthesis of *N*-hydroxysuccinimidyl benzoate and its structural insight into labeling activity of fluorescent probes

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Abstract

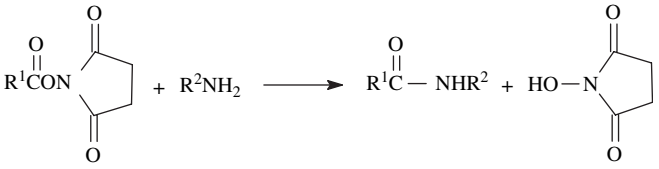
N-Hydroxysuccinimidyl benzoate was prepared as a model compound of fluorescent probes. Its crystal structure was determined. The interlayer space between neighbored aromatic rings is inserted by the carbonyl group of succinimidyl moiety and the stacking distance between aromatic rings is as far as 5.9085 Å. Labeling activity of carbonyl groups in *N*-hydroxysuccinimidyl benzoate was studied by AM1 semiempirical method and the result indicated that carbonyl group consisting of O4 and C5 was responsible for the labeling reaction center.

Keywords: *N*-Hydroxysuccinimidyl benzoate; X-ray; Crystal structure; AM1

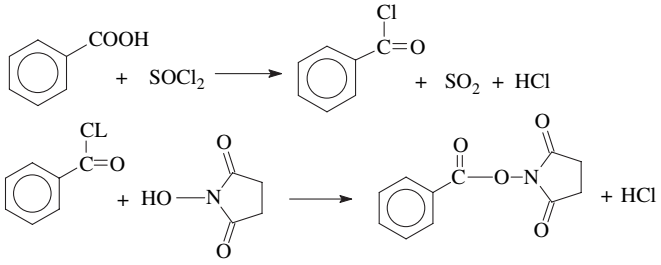
1. Introduction

Spectrofluorimetry is a simple, sensitive, and rapid method for the determination of bioactive molecules. Some dyes have been developed as fluorescent probes to increase the sensitivity of high performance liquid chromatography and capillary electrophoresis chromatography [1]. The *N*-hydroxysuccinimidyl ester was often designed as labeling group in structures of fluorescent probes [2,3] which could react with amino containing molecule (Scheme 1). Now the fluorescent probe plays an important role in detecting bioactive molecules in trace amounts [4].

Our investigation is to focus on the structure and reaction mechanism of fluorescent probes. As single crystals of *N*-hydroxysuccinimidyl esters containing dye moieties for X-ray



Scheme 1. Labeling reaction with amino containing molecule.



Scheme 2. Synthesis of *N*-hydroxysuccinimidyl benzoate.

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Table 1
Crystal data and experimental details

Compound	$C_{11}H_9NO_4$
Crystal dimensions (mm)	0.492 × 0.457 × 0.103
Crystal system	Monoclinic
Space group	$P2(1)/c$
Unit cell dimensions	$a = 11.0158(17)$ Å
	$b = 8.5273(14)$ Å
	$c = 11.0896(17)$ Å
	$\beta = 102.358(3)^\circ$
Volume (Å^3)	1017.6(3)
Z	4
Density (calculated) (g/cm^3)	1.431
Temperature (K)	293(2)
Wavelength (Å)	0.71073
θ Range for data collection	1.89–26.99°
Reflections collected	Total: 5749
	Unique: 2200 ($R_{int} = 0.0744$)
Refinement method	Full-matrix least-squares on F^2
Data/restraints/parameters	2200/0/182
Goodness-of-fit on F^2	0.774
Final R indices ($I > 2\sigma(I)$)	$R1 = 0.0368$, $wR2 = 0.0639$
R indices	$R1 = 0.0819$, $wR2 = 0.0729$
Extinction coefficient	0.0120(15)
Largest diff. peak and hole	0.116 and −0.121 e$^-$/Å^3

Table 2
Fractional atomic coordinates and equivalent isotropic displacement parameters

	x/a	y/b	z/c	U(eq)
O1	0.15365(12)	−0.05318(15)	0.61910(11)	0.0742(4)
O2	0.45859(12)	0.26157(15)	0.83176(11)	0.0784(4)
O3	0.23404(10)	0.11994(13)	0.83777(10)	0.0585(4)
O4	0.33482(12)	−0.09951(15)	0.91488(11)	0.0802(5)
N1	0.29617(13)	0.10442(16)	0.74227(12)	0.0518(4)
C1	0.24888(17)	0.0184(2)	0.63693(16)	0.0514(5)
C2	0.34199(18)	0.0384(3)	0.55723(17)	0.0541(5)
C3	0.4457(2)	0.1394(3)	0.62908(18)	0.0587(5)
C4	0.40789(18)	0.1810(2)	0.74704(15)	0.0540(5)
C5	0.26587(16)	0.0037(2)	0.92647(15)	0.0501(5)
C6	0.20294(15)	0.02995(18)	1.02903(14)	0.0443(4)
C7	0.23323(18)	−0.0709(2)	1.13010(17)	0.0571(5)
C8	0.1733(2)	−0.0560(3)	1.22556(19)	0.0716(6)
C9	0.0856(2)	0.0572(3)	1.2239(2)	0.0713(7)
C10	0.05629(19)	0.1583(3)	1.1254(2)	0.0641(6)
C11	0.11457(16)	0.1460(2)	1.02698(17)	0.0508(5)
H1	0.3672(15)	−0.0605(19)	0.5390(14)	0.060(5)
H2	0.3008(15)	0.0855(18)	0.4810(16)	0.070(6)
H3	0.4607(15)	0.232(2)	0.5866(15)	0.080(6)
H4	0.5179(16)	0.0788(18)	0.6517(15)	0.067(6)
H5	0.2932(15)	−0.149(2)	1.1262(15)	0.073(6)
H6	0.1932(15)	−0.1243(19)	1.2922(15)	0.069(6)
H7	0.0437(18)	0.0615(19)	1.2914(16)	0.092(7)
H8	−0.0021(15)	0.2393(19)	1.1187(14)	0.064(6)
H9	0.0893(13)	0.2145(17)	0.9538(12)	0.055(5)

diffraction technique are difficult to obtain, *N*-hydroxysuccinimidyl benzoate was synthesized as a model compound of fluorescent probes (Scheme 2). Subsequently, the crystal structure and labeling activity of *N*-hydroxysuccinimidyl benzoate was studied, too.

2. Experimental

2.1. Synthesis of N-hydroxysuccinimidyl benzoate

Benzoic acid (1.2 g; 0.01 mol) was placed in a 150 mL Erlenmeyer flask fitted with a reflux condenser. With agitation, 20 mL thionyl dichloride ($SOCl_2$) was added to the flask slowly. The resulting HCl gas was absorbed by NaOH solution. Further agitation was continued at the reflux temperature for 3 h. The excess $SOCl_2$ was removed from the reaction mixture by vacuum evaporation. *N*-hydroxysuccinimide (1.2 g) in 20 mL ethyl acetate was then added to the flask slowly and followed by agitation for 3 h at reflux temperature. As cooled to room temperature, the reaction mixture was washed with $NaHCO_3$ solution and water. The oil layer was then separated and dried by Na_2SO_4. After vacuum distillation of the solvent and recrystallization by ethyl acetate–ethyl ether, the pure product was obtained as white crystals in a yield of 65%. UV–vis (nm): 243, 278. IR (KBr/cm^{-1}): 1736, 1776, 1072, 998. ^{1}H NMR ($CDCl_3/\delta$, ppm): 8.14 (2H, m), 7.67(1H, m), 7.54(2H, m), 2.91(4H, s).

2.2. Preparation of a single crystal

Slow evaporation of ethyl acetate solution of *N*-hydroxysuccinimidyl benzoate gave colorless crystals after several

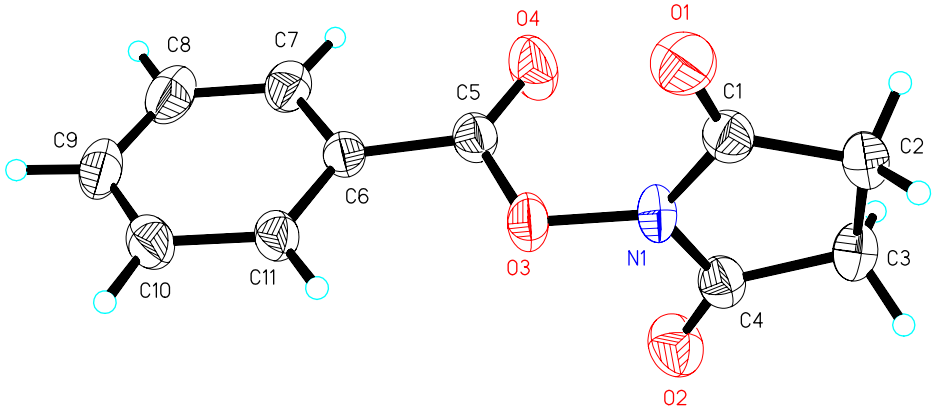

Fig. 1. Molecular structure and atomic numbering of *N*-hydroxysuccinimidyl benzoate.

Table 3
Dihedral angles between planes (x, y, z in crystal coordinates)

Plane	Equation	Angle to previous plane
1	$4.4401x - 6.9109y + 3.6490z = 3.2975$	
2	$6.9152x + 5.3937y + 3.4249z = 5.0952$	86.18°

days. The single crystal was collected by filtration and used in the following investigation.

2.3. X-ray crystal analysis

X-ray diffraction data were collected at 293 K on a Bruker SMART diffractometer with graphite monochromated Mo Kα radiation ($\lambda = 0.71073$ Å). The crystal structure was solved and refined with standard techniques [5,6]. All U values of H-atoms were refined to realistic values. Relevant numerical data are given in Table 1 and the final fractional atomic coordinates are listed in Table 2.

2.4. Computational approach

The study was carried out using AM1 semiempirical method in the commercially available computer program package (HyperChem Pro. Release 6.03 from Hypercube Inc. USA). The molecular geometry was based on the result of X-ray crystal analysis.

3. Results and discussion

3.1. Crystal structure of N-hydroxysuccinimidyl benzoate

A general view of one molecule of *N*-hydroxysuccinimidyl benzoate is shown in Fig. 1. The molecule contains an aromatic ring and a 5-member succinimidyl ring. Results of calculations of least-squares planes are given in Table 3, and indicate that each ring keeps its planarity. The planarity of succinimidyl ring is also confirmed by the single peak of hydrogen atoms connected to C2 and C3 in ^{1}H NMR spectrum. The angle between the planes of the aromatic ring and succinimidyl ring is 86.18°, which is due to the repelling forces between carbonyl groups. Crystal packing scheme is shown in Fig. 2. The aromatic rings are stacked in the direction of *b*-axis, but the interlayer space between neighbored aromatic rings is inserted by the carbonyl group in succinimidyl moiety, which decreases the π–π electron interaction between aromatic rings. The stacking distance between planes of the neighboring aromatic rings is as far as 5.9085 Å.

3.2. Labeling activity of carbonyl groups in N-hydroxysuccinimidyl benzoate

According to reaction mechanism, the carbonyl group acts as the substrate in labeling reaction of active esters. Generally, the O atom in carbonyl group is the site of electrophilic attack and the C atom is the site of nucleophilic attack. The reaction activity of carbonyl group is dominated by charge density of the attack sites. There are three carbonyl groups in the molecular structure

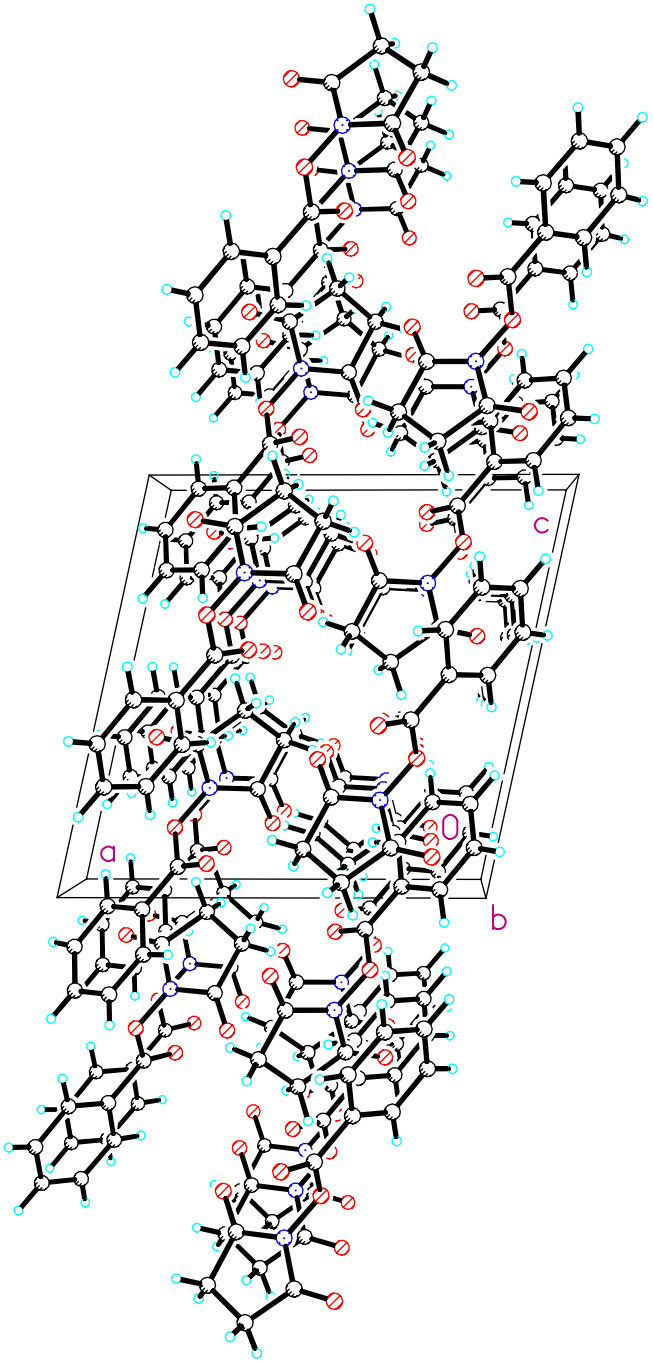

Fig. 2. Crystal packing scheme of *N*-hydroxysuccinimidyl benzoate.

of *N*-hydroxysuccinimidyl benzoate. Charges on the atoms of carbonyl groups are shown in Table 4, which were obtained using AM1 semiempirical method in HyperChem program package. The calculation result clearly indicates that the O4 has the most negative charge of −0.383 and C5 has the most positive

Table 4
Charges on the atoms in carbonyl groups

	Positive charge		Negative charge	
C5═O4	C5	0.468	O4	−0.383
C1═O1	C1	0.374	O1	−0.368
C4═O2	C4	0.372	O2	−0.366

charge of 0.468, which means that carbonyl group consisting of O4 and C5 is responsible for the labeling reaction center of *N*-hydroxysuccinimidyl active ester.

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