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Azobenzene derivatives show anti-cancer activity against pancreatic cancer cells only under nutrient starvation conditions *via* G_0/G_1 cell cycle arrest

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ABSTRACT

Pancreatic cancer is one of the most aggressive cancers with a poor prognosis. Previous studies suggested that nutrient-deprived conditions may play a critical role in pancreatic cancer cell survival and resistance to chemotherapy. We describe a novel series of azobenzene derivatives including *(E)*-1-(4-methyl-3-((2-methyl-5-(naphthalen-1-yl)phenyl)diazenyl)phenyl)naphthalen-2-ol (**9**) with efficacy and selectivity in nutrient-deprived conditions. Although anticancer drug 5-fluorouracil (5-FU) was ineffective under nutrient-deprived conditions, five of our designed compounds, **9** and four other related compounds **11**−**14**, showed anticancer activity with IC_{50} values ranging from 1.5 to 9.6 μM. Interestingly, only **9** showed no cytotoxicity in normal conditions. This selectivity profile of **9** is clearly opposite to that of 5-FU. Furthermore, cell cycle analysis showed that, in contrast to S phase arrest induced by 5-FU, **9** caused G_0/G_1 phase arrest, which might block cancer cell growth by arresting them in quiescence. Therefore, it could be a novel and promising candidate for effective pancreatic cancer treatment under nutrient-deprived conditions.

1. Introduction

Pancreatic cancer is one of the most aggressive cancers with poor prognosis [1]. A recent study has reported that a 5-year survival rate of pancreatic cancer patients was approximately 9% [2]. Moreover, in the case of pancreatic cancer, anticancer agents have only limited effects in clinical practice [3]. Also, a recent clinical trial has shown that a median overall survival with gemcitabine (GEM), which is widely used for chemotherapy of advanced pancreatic cancer, was just 6.8 months [4]. Hence, the treatment of pancreatic cancer is one of the most critical challenges, and a new anti-pancreatic-cancer agent is sorely needed to improve treatment outcomes.

To further elucidate the biological features of pancreatic cancer, several studies have focused on metabolic changes and adaptations under nutrient-deprived conditions. These studies have revealed that pancreatic cancer displays the following unique features: (i) some cells are exposed to nutrient-deprived conditions in the tumor [5], (ii) the cells can survive under the nutrient-deprived medium (NDM), which is inclusive of electrolytes and vitamins but exclusive of glucose, amino acids, and serum [6,7], and (iii) under such conditions, the cells exhibit resistance to anticancer drugs like 5-fluorouracil (5-FU) and GEM [8]. Recent studies have also shown that autophagy, which is an intracellular degradation and recycling process induced by nutrient deprivation or cell stress, is highly upregulated in pancreatic cancer [9] and may contribute to survival under nutrient-deprived conditions [10,11]. These features may be associated with poor prognoses [12]. It appears that nutrient-deprived conditions may play a critical role in pancreatic cancer cell survival and resistance to chemotherapy. This is a difficult but important problem that needs to be addressed to improve clinical outcomes. Thus, we focused on nutrient-deprived

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conditions as a potential target for chemotherapy.

Previous studies have demonstrated that some compounds, including arctigenin [13] that has progressed to a clinical trial [14], showed anticancer activity under nutrient-deprived conditions [15–18]. To date, however, no drugs have been approved for use against nutrient-deprived conditions. With this in mind, we therefore aimed to investigate the effect of various synthesized compounds under nutrient-deprived conditions. In addition to efficacy, selectivity is also evaluated by the use of normal culture medium to avoid cytotoxic side effects in normal cells. Several derivatives were then selected for further evaluation after initial screening of the synthesized azobenzenes. Several previous studies have shown an azobenzene-containing polymer is a carrier to deliver anticancer agents into pancreatic cancer tissues [19,20]. Additionally, some azobenzene derivatives have been reported to show photo-switching ability of the biological activities by photo-irradiation based on photo-isomerizations [21,22]. However, anti-pancreatic cancer activity of azobenzene derivatives themselves has not been reported previously. This paper describes the synthesis and preliminary evaluation of a novel series of azobenzene derivatives (Fig. 1); the dimeric azonapthol (**9**) shows significant anti-pancreatic cancer activity, with both efficacy and selectivity in nutrient-deprived conditions.

2. Results and discussion

2.1. Synthesis of azobenzene derivatives

A series of azobenzene compounds with acid and/or base functionalities (**1**–**14**) were synthesized following the routes described in Schemes 1–4. The symmetrical azo derivative **1**, prepared from coupling of the diazonium salt of **15** as previously described [23] in 51% yield, was the divergent intermediate for the synthesis of azocompounds **2**–**7** (Scheme 1). All the initial azo compounds were isolated as mixtures of *cis*- and *trans*-isomers which gave the pure *trans*-isomer when warmed at 50 °C overnight.

As previously described [23], addition of phenylmagnesium bromide and of 1-naphthyllithium to the ester **1** gave the tertiary alcohols **2** and **3** in yields of 94% and 51% respectively. Reduction of the esters in **1** by di-isobutylaluminum hydride afforded **4** (84% yield); subsequent bromination of the primary alcohol **4** with tetrabromomethane and triphenyl phosphine provided the bromide **5** (74% yield). The amino azobenzene **6** was synthesized from **1** *via* initial sodium hydroxide hydrolysis to the acid **16** (85%); treatment of the acid **16** with diphenylphosphoryl azide in the presence of benzyl alcohol resulted in a Curtius rearrangement to give the carbamate protected amine **17** (69%). Basic solvolysis of the carbamate **17** (92%) gave the amino azobenzene **6** in an overall yield of 54% from **1**. Reductive *N*-methylation of **6** by treatment with formaldehyde and sodium borohydride gave the methylamino azobenzene **7** in 42% yield.

When the bromide **20** was treated with the amino boronic acid **21** in the presence of $Pd(PPh_3)_4$, the biphenyl azo-amine **8** was isolated in 89% yield. Similar $Pd(PPh_3)_4$-catalysed coupling of the bromide **20** with 2-methoxy-1-naphthaleneboronic acid **22** gave the methoxy naphthalene **23** (85% yield) which was demethylated on treatment with boron tribromide to give the naphthols **9** (92%).

A similar strategy was successful in the synthesis of a diphenylphosphine azobenzene **10** (Scheme 3). Condensation of the iodoaniline **24** with the nitroso compound **19** [derived from **18**] gave the bromo iodoazo analogue **25** (73%). The iodide in **25** was selectively replaced by treatment with a mixture of diphenylphosphine and diphenylphosphine oxide in the presence of palladium acetate to give the phosphine oxide **26** (77% yield). Coupling of the bromide **26** with naphthalene boronic acid **22** in the presence of a catalytic amount of $Pd(PPh_3)_4$ formed the naphthalene derivative **27** in quantitative yield. Removal of the methyl ether in **27** by treatment with boron tribromide gave the naphthol **28** (70%). Reduction of the phosphine oxide group in **28** by trichlorosilane gave the target phosphine **10** (24%) in an overall yield of 13% from **25**.

The amino azobenzene **6** was a divergent intermediate for the efficient synthesis of a series of azobenzene derivatives **11**–**14** containing both pyridine and thiourea or urea moieties (Scheme 4). The pyridine units were introduced by a palladium-catalysed amination reaction; the thiourea or urea units were introduced by the reactions of amines with isothiocyanate or isocyanate. The azo compound **6** was coupled with the iodo-dimethylaminopyridine **29** in the presence of Pd_2dba_3 and 1,3-bis(diphenylphosphino)propane to give the DMAP analogue **30** (47% yield). Subsequent treatment with the *bis*(trifluoromethyl) phenyl isothiocyanate **31** gave the thiourea **11** (50%), or with the corresponding isocyanate **32**, afforded the urea **12** (42% yield).

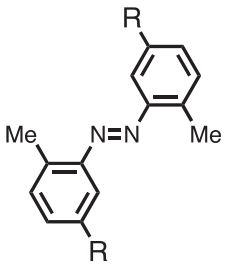
Palladium-catalysed coupling of **6** with 2-iodopyridine **33** formed the pyridine analogue **34** (46%); subsequent reaction of **34** with the isothiocyanate **31** gave the corresponding thiourea **13** (57%). Similar coupling of **6** with the pyrrolidine **35** gave the amine **36** (42%); treatment of **36** with the isothiocyanate **31** afforded **14** in 40% yield.

2.2. Evaluation of anti-cancer activity of azobenzene derivatives

The first aim of our study was to determine the efficacy of our compounds under nutrient-deprived conditions. Initially, we evaluated anticancer activity of nine azobenzene derivatives (**1**–**9**), 5-fluorouracil (5-FU), and gemcitabine (GEM) against the human pancreatic cancer cell line PANC-1 [24] under nutrient-deprived conditions by using nutrient-deprived medium (NDM), which is exclusive of glucose, amino acids, and serum. Cell viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [25]. Table 1 shows IC_{50} values for the tested compounds.

Reference anticancer drugs 5-FU and GEM showed no activity even at 200 μM (Fig. 2A and B). Several studies have suggested mechanisms for resistance to current anticancer drugs under nutrient-deprived conditions [26]. One example is glucose-regulated protein 78 (GRP78) which is one of the molecular chaperones and has been proposed as a possible factor contributing to chemotherapy resistance [27]. It is not clear, however, which factors responsible for this phenomenon. Our results and previous studies highlight the issues of current anticancer drugs that lack activity under nutrient-deprived conditions. In contrast to the results of 5-FU and GEM, synthesized compound **9** showed anticancer activity under nutrient-deprived conditions ($IC_{50} = 4.1 \pm 0.3$ μM). In light of this result, we further evaluated five additional azobenzene derivatives (**10**–**14**). These results demonstrated that four related compounds (**11**–**14**) showed anticancer activity with IC_{50} values ranging from 1.5 to 9.6 μM under nutrient-deprived conditions. Taken together, we identified five azobenzene derivatives (**9**, **11**, **12**, **13**, and **14**) that showed potent efficacy under nutrient-deprived conditions, where the current anticancer drugs are not effective. Our findings have also opened up possibilities of using azobenzene derivatives for drug discovery in pancreatic cancer.

We next evaluated safety profiles of azobenzene derivatives to minimize cytotoxicity under normal conditions. In clinical practice, chemotherapy-related side effects, such as myelosuppression, diarrhea, and neurotoxicity, are serious problems. These side effects are caused by cytotoxic effects against normal cells [28–30]. Hence, in addition to anticancer activity, minimizing cytotoxicity against normal cells is required for safe and effective treatment. In the



No.	R
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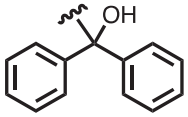
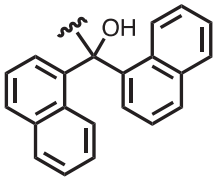
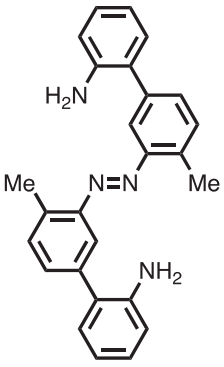
2	
3	
4	-CH_2OH
5	-CH_2Br
6	-NH_2
7	-$NHCH_3$



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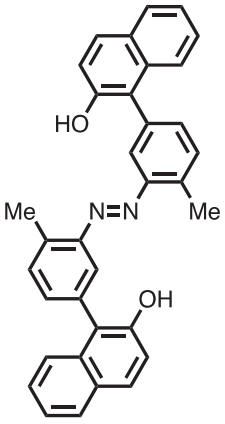

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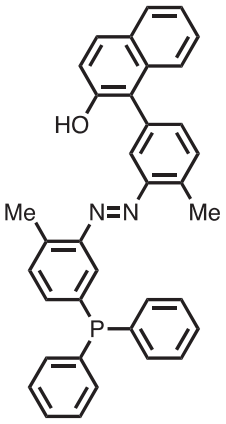

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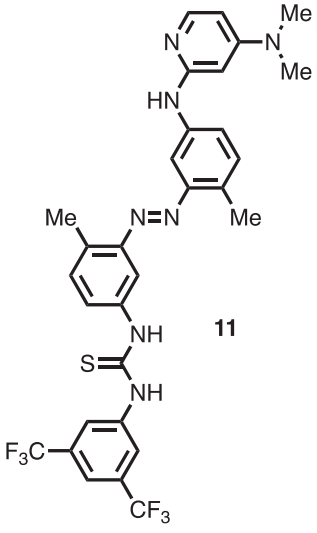

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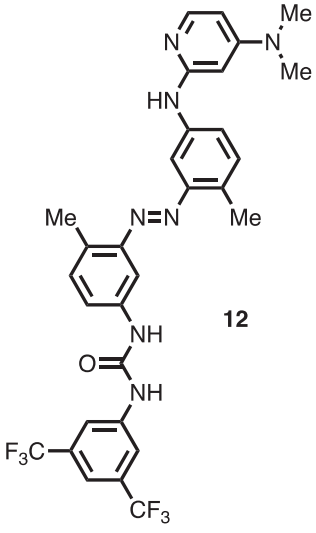

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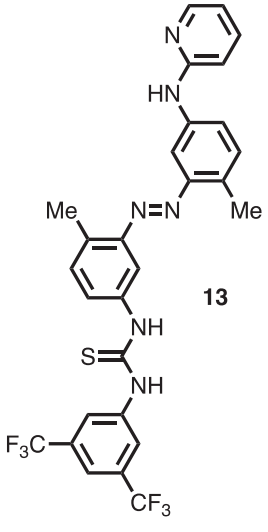

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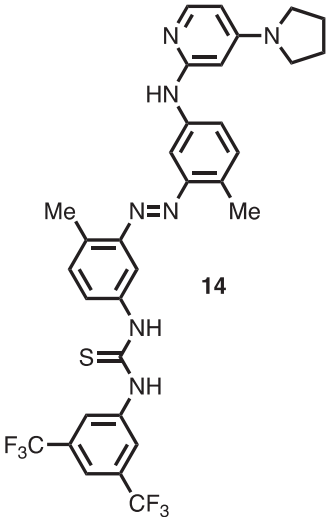

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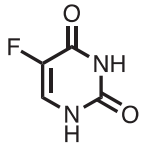
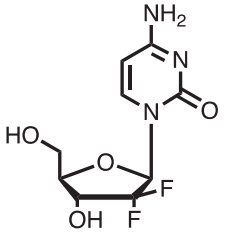
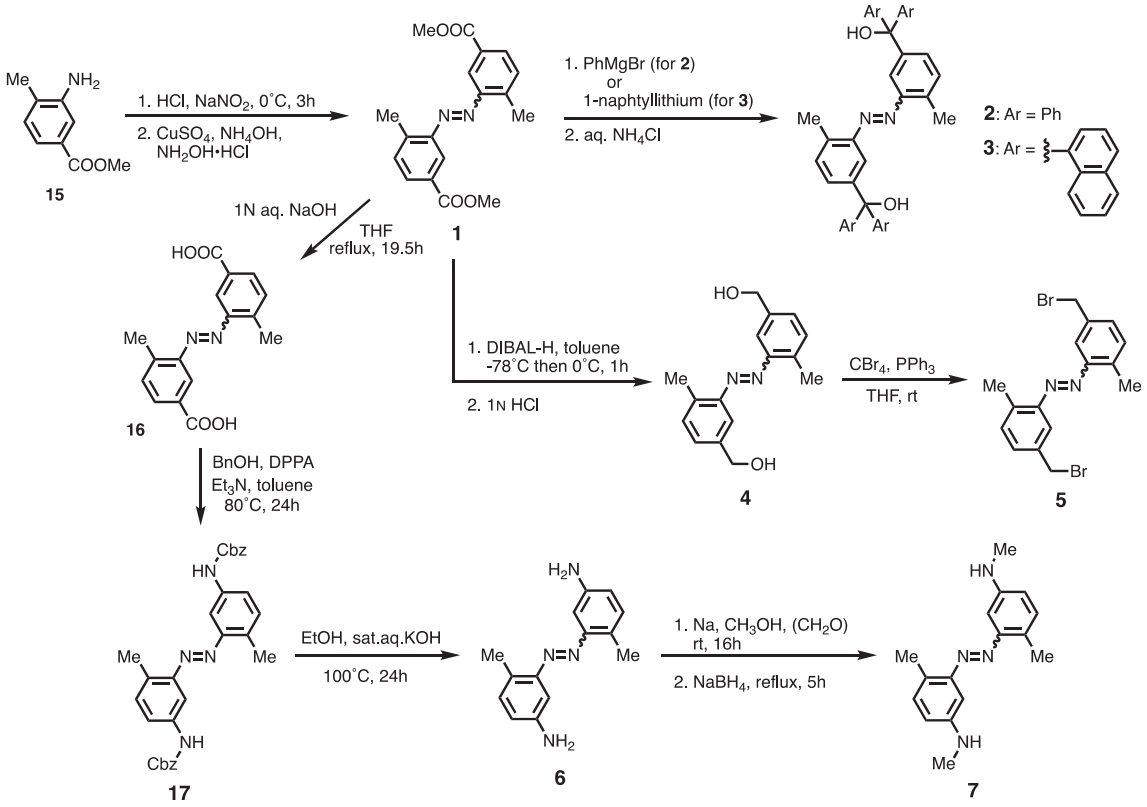


5-fluorouracil
5-FU

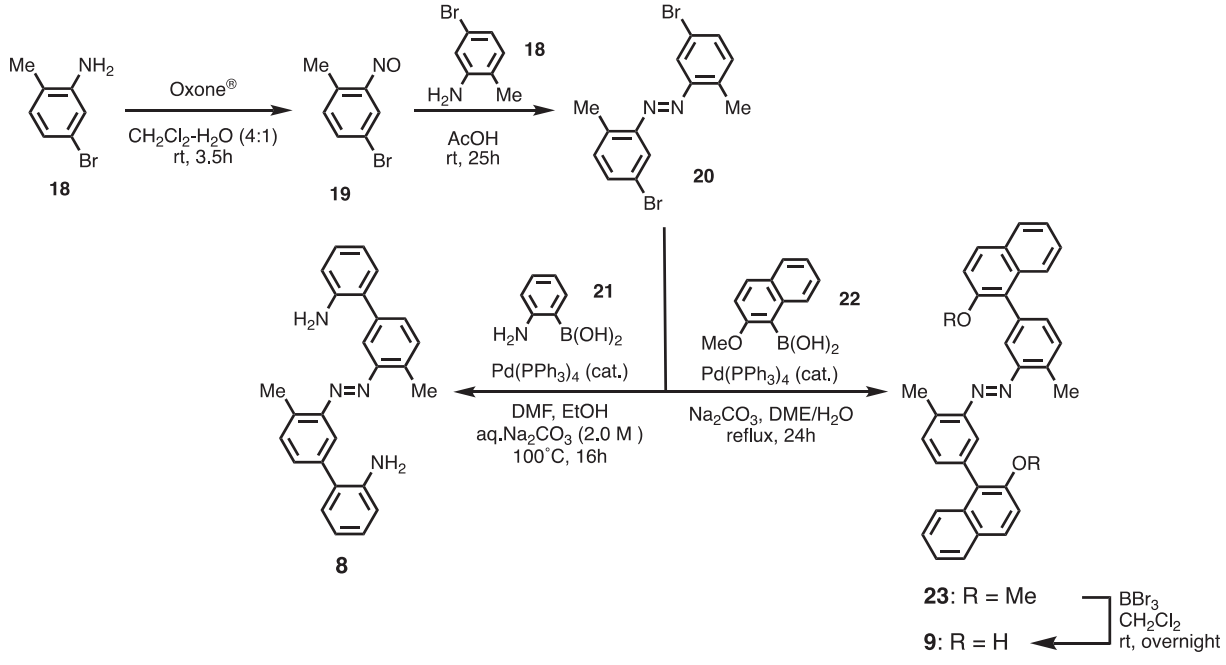


Gemcitabine
GEM

Fig. 1. Structures of the azobenzene derivatives **1**–**14** and anti-cancer compounds 5-FU and GEM.



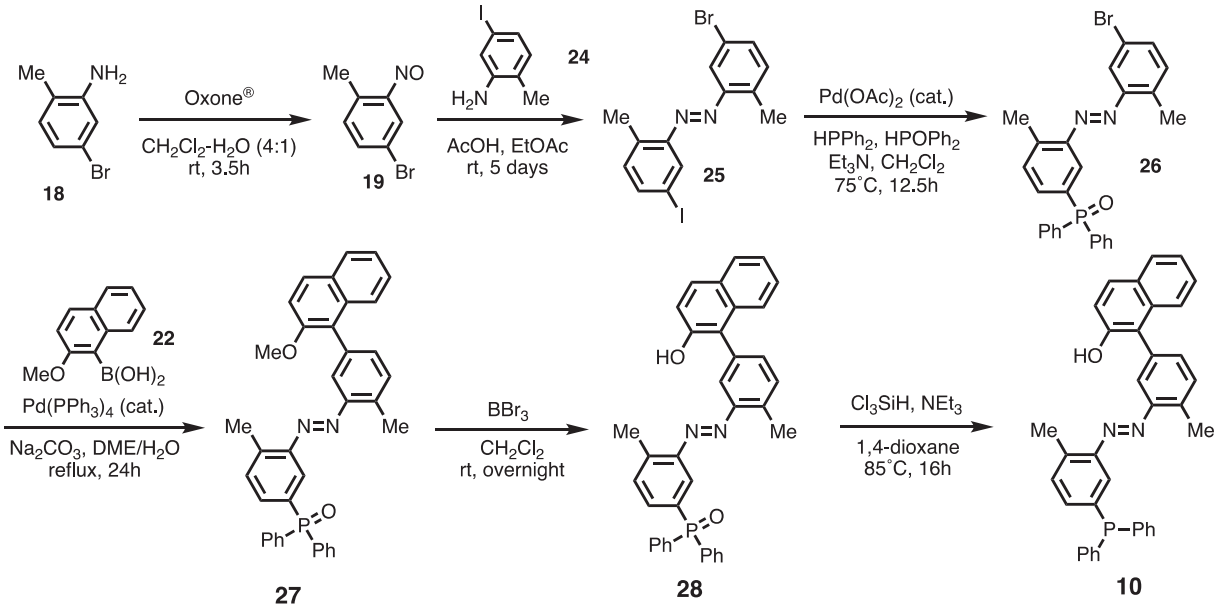
Scheme 1. Synthesis of azobenzenes **1**–**7**.



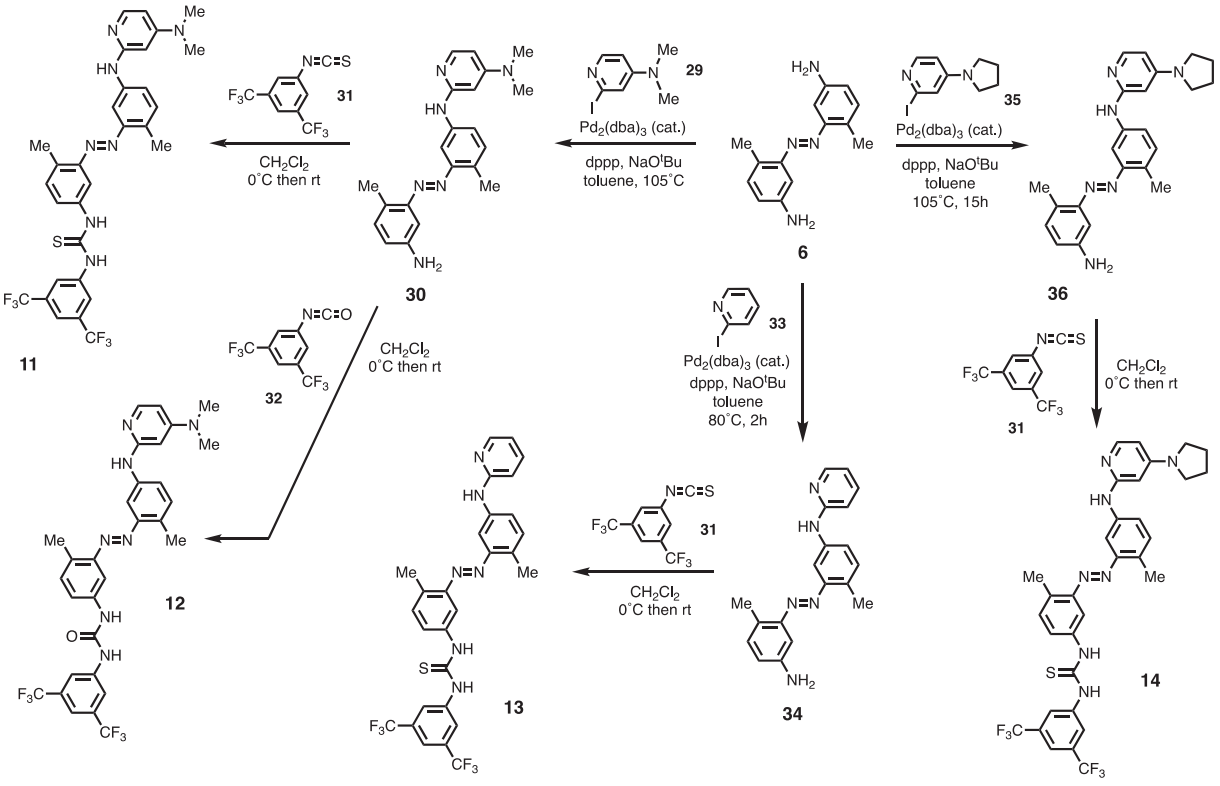
Scheme 2. Pd-catalysed coupling in the synthesis of azobenzenes **8** and **9**.

present study, we focused on nutrient-deprived conditions where pancreatic cancer cells can survive, but not normal cells. Thus, because normal cells cannot exist under such conditions, selective targeting of nutrient-deprived conditions may contribute to reducing toxicity against normal cells.

In light of these points, we examined whether their activities are selective against nutrient-deprived conditions, by assessing their cytotoxicity under normal conditions by using normal medium (Dulbecco's modified Eagle's medium; DMEM). Unfortunately, most of these compounds showed cytotoxicity under normal conditions



Scheme 3. Pd-catalysed coupling in the synthesis of a diphenylphosphine azobenzene **10**.



Scheme 4. Amino azobenzene **6** as a divergent intermediate for pyridine azobenzenes **11**–**14**.

(IC_{50} values ranging from 7.1 to 16.9 μM) as well as anticancer activity under nutrient-deprived conditions, but interestingly, only **9** showed no cytotoxicity under normal conditions (Fig. 2C). We also tested the toxicity of **9** against the human normal fibroblast cell line GM05659 under normal conditions. In normal cells, **9** did not affect the cell viability even at 20 μM (Fig. 3). These results indicated that **9** exhibits selective activity against nutrient-deprived conditions with both potency and safety.

In light of these results, we investigated the importance of the naphthol unit in **9** and **10**. Compound **9** has naphthol units on both sides of the azobenzene core and shows anticancer activity only under nutrient-deprived conditions. Compound **10** with only the naphthol unit on one side does not show such activity both under nutrient-deprived and normal conditions. These results suggested that a combination of the naphthol unit may be important for selective activity. In this study we elucidated that compounds **11**–**14**

Table 1
IC_{50} values of test compounds (**1**–**14**, 5-fluorouracil, and gemcitabine) against PANC-1 cells under normal conditions and nutrient-deprived conditions.

No.	IC_{50} [μM]	
	Normal conditions	Nutrient-deprived conditions
	72 h	24 h
1	>80	>80
2	>80	>80
3	>80	>80
4	>80	>80
5	>80	>80
6	>80	>80
7	>80	>80
8	>80	>80
9	>80	4.1 ± 0.3
10	>80	>80
11	7.1 ± 0.3	1.5 ± 0.1
12	14.3 ± 1.3	3.6 ± 0.5
13	16.9 ± 0.4	5.4 ± 0.8
14	7.7 ± 0.4	9.6 ± 0.1
5-FU	3.2 ± 0.3	>200
GEM	23.5 ± 3.1	>200

Data are presented as mean ± SE ($n = 3$). 5-FU; 5-fluorouracil, GEM; gemcitabine.

showed powerful anticancer effect under both normal conditions and nutrient-deprived conditions. They have a combination of DMAP (4-dimethilaminopyridine) and thiourea or related structures. In light of the activity profiles, these combinations may be important for the powerful but non-selective activity of **11**–**14**. Further studies with different modification of the napthol moiety may produce more selective agents.

To further characterize the anticancer activity of **9**, we next performed a cell cycle analysis by propidium iodide staining [31]. PANC-1 cells were treated with or without **9** under either normal or nutrient-deprived conditions for 24 h, then stained with propidium iodide and analyzed. Treatment with compound **9** significantly increased the percentage of cells in G_0/G_1 phase (47.5 ± 1.0%, $p < 0.01$ vs control cells) and decreased those in S phase (18.7 ± 0.6%, $p < 0.01$ vs control cells) under nutrient-deprived conditions (Fig. 4A). In contrast, **9** did not affect the cell cycle under normal conditions even at 100 μM (Fig. 4B). There are possible targets, including CDK4/6, PI3K/AKT, p21, that lead to cell cycle arrest in G_0/G_1 phase. These factors might play a role in selective activity under nutrient-deprived conditions. In light of this, further research focused on these factors is needed to elucidate the mechanisms underlying our findings [32,33].

These results demonstrated two key points: (i) the effect of **9** on the cell cycle was selective in nutrient-deprived conditions as was its anticancer activity, and (ii) **9** caused G_0/G_1 phase arrest, which is a different mode of action to that of S phase arrest induced by 5-FU [34]. The anticancer activity results and cell cycle arrest may well have a related mechanism. Although further detailed study is needed, one possibility is that **9** might block cancer cell growth by arresting cells in G_0 phase, which is a state of cellular quiescence [35].

3. Conclusion

In conclusion, five of the designed azobenzene showed significant anticancer activity under nutrient-deprived conditions. These findings provide unprecedented insight into the anticancer activity of azobenzene derivatives. The symmetrical azodinapthol derivative **9** differed from the other four compounds in selectivity against nutrient-deprived conditions. This selectivity of **9** is likely to be important in the reduction of toxicity to normal cells under normal conditions. Furthermore, **9** showed G_0/G_1 phase arrest, in contrast to S phase arrest induced by 5-FU. The effect of **9** on cell cycle progression was also selective against nutrient-deprived conditions. Thus, the synthesized azobenzene derivative **9** could be a novel and promising candidate for effective and selective pancreatic cancer treatment under nutrient-deprived conditions.

4. Experimental section

4.1. Chemistry

4.1.1. General

In each synthesis, a mixture of both the *cis*- and *trans*-azobenzenes was initially formed; however when the mixture was warmed at 50 °C overnight, pure *trans*-azobenzene isomers were isolated. The thermal isomerization of *cis*-to *trans*-azobenzenes under these conditions is well-established [36]. NMR spectra were recorded on JEOL JNM-EX 400 (400 MHz) spectrometer, Varian INOVA 400 (400 MHz), BRUKER BioSpin-AVANCE DPX-400 (400 MHz), BRUKER BioSpin-AVANCE DPX-300 (300 MHz), and BRUKER BioSpin-AVANCE 400 M (400 MHz). ^{13}C NMR spectra were recorded using broad band proton decoupling. Residual $CHCl_3$ peak or tetramethylsilane (TMS) were used as an internal reference for ^1H NMR ($CHCl_3$: 7.26 ppm, TMS: 0.00 ppm). For ^{13}C NMR in $CDCl_3$, residual peak of $CHCl_3$ was used as an internal reference (77.16 ppm). Chemical shifts are expressed in δ (ppm) values, and coupling constants are expressed in hertz (Hz). The following abbreviations are used: s = singlet, d = doublet, m = multiplet, br s = broad singlet, dt = double-triplet, and dd = double-doublet.

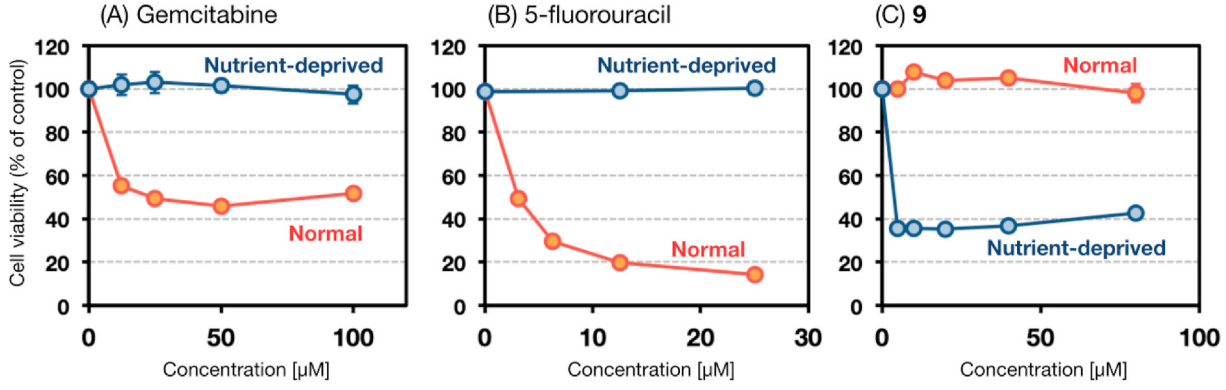


Fig. 2. Anticancer activities of (A) gemcitabine, (B) 5-fluorouracil, and (C) **9** against PANC-1 cells under normal conditions and nutrient-deprived conditions. Results are expressed as mean ± SEM ($n = 3$).

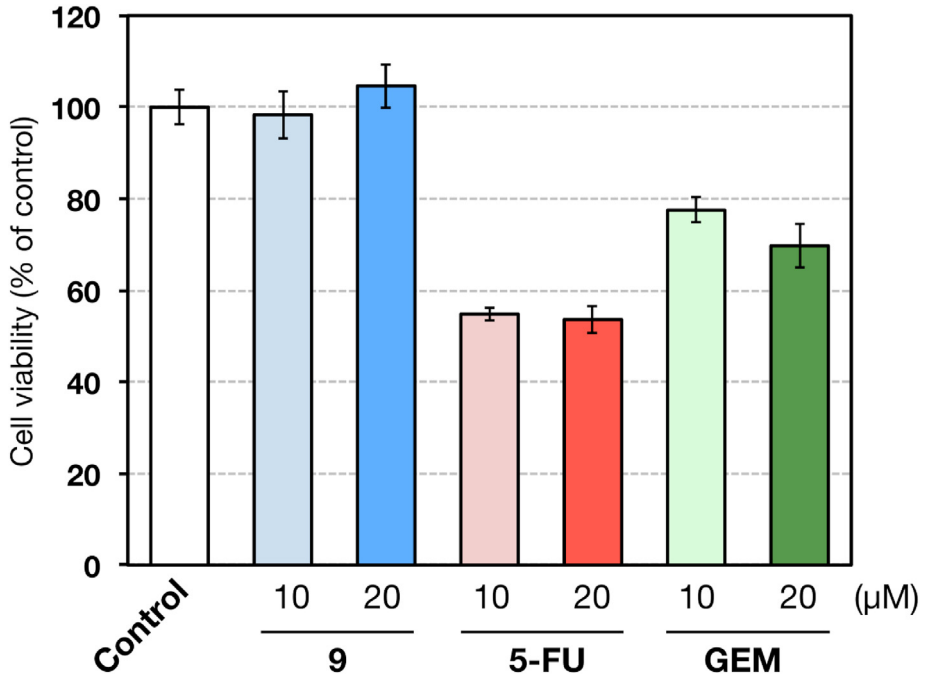


Fig. 3. Toxicity of **9**, 5-fluorouracil (5-FU), and gemcitabine (GEM) against GM05659 cells under normal conditions. GM05659 cells were cultured with or without compound for 72 h. Results are expressed as mean ± SEM ($n = 3$).

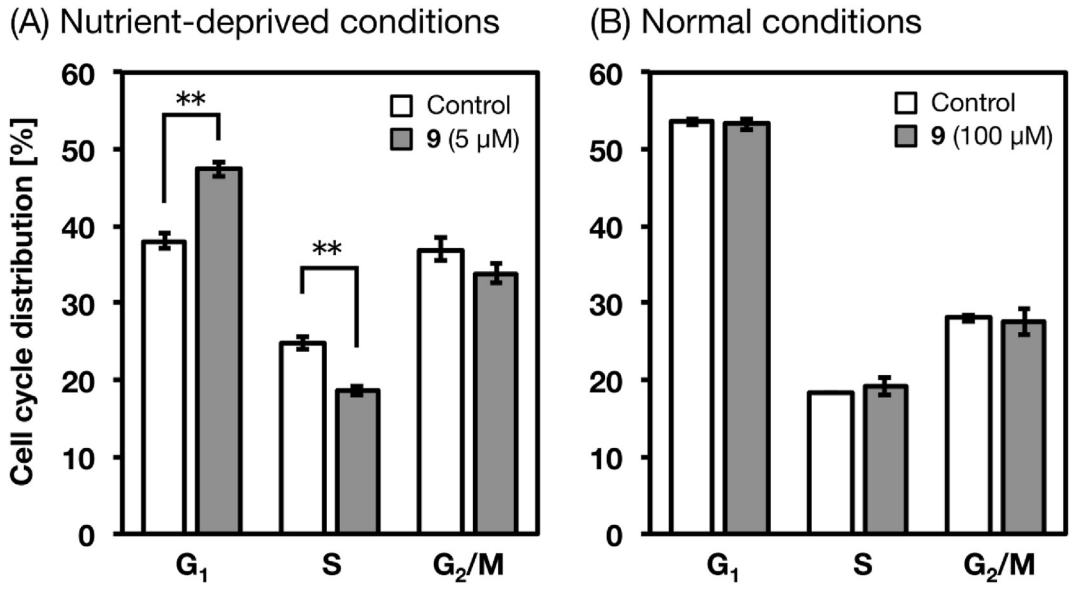


Fig. 4. The cell cycle analysis. PANC-1 cells were cultured with or without **9** in (A) nutrient-deprived conditions or (B) normal conditions for 24 h. Statistical significance was determined by using Welch's *t*-test (**, $p < 0.01$ vs control cells). Each value represents the mean ± SEM ($n = 3$).

Mass spectra and high-resolution mass spectra were recorded on a JEOL JMS-700, Varian 910-MS FT-ICR. IR spectra were measured with a PerkinElmer spectrum one S series FT-IR spectrometer and a JASCO FT-IR 466ST. All reagents and solvents are commercial grade and were used as supplied without further purification. Known compounds were identified by ^{1}H NMR and ^{13}C NMR spectra.

*4.1.2. Dimethyl 3,3'-(diazene-1,2-diyl)(E)-bis(4-methylbenzoate)**1** [23]*

Methyl 3-amino-4-methylbenzoate **15** (16.0 g, 96.9 mmol) in 7.4% aqueous HCl (200 mL) was added dropwise to a solution of sodium nitrite (7.04 g, 102 mmol) in water (600 mL) over 2 h 50 min at 0 °C to form a solution of the corresponding diazonium salt of **15** for use in the next step.

An aqueous solution of $CuSO_4 \cdot (H_2O)_5$ (28.5 g, 114 mmol) in water (100 mL) was treated with 30% aqueous ammonia (30 mL) at 0 °C; the reaction mixture was allowed to warm to room temperature. Then an aqueous solution of $NH_2OH \cdot HCl$ (8.0 g, 115 mmol in water (20 mL) was added until the deep blue color disappeared, after which the solution of diazonium salt of **15** was added dropwise and the reaction mixture was stirred for 1 h. The resulting dark orange solid was collected by filtration, washed with distilled water

and dried under reduced pressure. The crude product was purified by silica gel column chromatography (CH_2Cl_2 as eluent) and crystallization (CH_2Cl_2/MeOH) to afford **1** as an orange solid (8.05 g, 24.7 mmol, 51%, mixture of *trans*- and *cis*-azobenzene isomer) which was warmed at 50 °C overnight to afford the pure *trans*-azobenzene isomer **1**.

^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 2.82 (s, 6H), 3.94 (s, 6H), 7.44 (d, 2H, J = 8.0 Hz), 8.05 (dd, 2H, J = 7.9, 1.8 Hz), 8.23 (d, 2H, J = 1.7 Hz). ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm): 18.0, 52.2, 117.2, 128.7, 131.4, 131.5, 143.4, 150.7, 166.8.

4.1.3. (E)-(Diazene-1,2-diylbis(4-methyl-3,1-phenylene)) bis(diphenylmethanol) **2** [23]

A 1.0 M THF solution of phenylmagnesium bromide (8.8 mL, 8.8 mmol) was added dropwise at 0 °C under an argon atmosphere to a solution of a mixture of *cis*- and *trans*-azobenzene isomers **1** (652 mg, 2.0 mmol) in THF (8.8 mL). The reaction mixture was then stirred at 30 °C for 26 h, quenched by the addition of saturated aqueous solution of NH_4Cl, and extracted with dichloromethane. The organic extracts were dried (Na_2SO_4) The solvent was removed under reduced pressure and the residue was purified by silica gel column chromatography (CH_2Cl_2/hexane = 2/1) to afford a mixture of *trans*- and *cis*-azobenzene isomers **2** (1.08 g, 1.89 mmol, 94%) which was warmed at 50 °C overnight to afford the pure *trans*-azobenzene isomer **2**.

^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 2.53 (br s, 6H), 2.83 (br s, 2H), 7.28 (br s, 24H), 7.47 (br s, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm): 17.1, 81.9, 115.4, 127.3, 127.9, 128.0, 130.2, 130.9, 137.1, 145.2, 146.7, 150.2.

4.1.4. (E)-(Diazene-1,2-diylbis(4-methyl-3,1-phenylene)) bis(di(naphthalen-1-yl)methanol) **3** [23]

A solution of 1-naphthyllithium was prepared by addition of 1.62 M solution of nBuLi (6.17 mL, 10.0 mmol) to 1-bromonaphthalene (1.46 mL, 2.16 g, 9.91 mmol) in THF at −78 °C under an argon atmosphere and the mixture was stirred at −78 °C for an additional 30 min.

To the above solution of 1-naphthyllithium was added a mixture of *cis*- and *trans*-azobenzene isomers **1** (652.3 mg, 2.0 mmol) in THF (20 mL) dropwise at −78 °C under argon. The reaction mixture was allowed to warm to −10 °C and stirred for 1 h and the reaction quenched by addition of sat. aq. solution of NH_4Cl and extracted with dichloromethane. The organic extracts were dried (Na_2SO_4) and the solvent removed under reduced pressure to give a residue which was purified by silica gel column chromatography (hexane/CH_2Cl_2 = 1/1) to afford a mixture of *trans*- and *cis*-azobenzene isomers **3** (795 mg, 1.0 mmol, 51%) which was warmed at 50 °C overnight to afford the pure *trans*-azobenzene isomer **3**.

^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 2.46 (s, 6H), 3.65 (s, 2H), 6.87 (d, J = 6.8 Hz, 4H), 7.18–7.28 (m, 12H), 7.39 (d, J = 7.8 Hz, 2H), 7.41 (d, J = 6.8 Hz, 2H), 7.68 (br s, 2H), 7.80 (d, J = 8.8 Hz, 4H), 7.86 (d, J = 7.8 Hz, 4H), 8.28 (d, J = 7.8 Hz, 4H). ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm): 17.1, 85.2, 115.2, 124.3, 125.4, 125.6, 128.3, 128.6 (br m), 128.881, 129.3, 130.1, 130.9, 131.3, 135.1, 137.2, 141.9 (br m), 145.3, 150.4.

4.1.5. (E)-(Diazene-1,2-diylbis(4-methyl-3,1-phenylene)) dimethanol **4**

A solution of di-isobutylaluminum hydride (1.0 M hexane, 6.8 mL, 6.8 mmol) was added to a mixture of *cis*- and *trans*-azobenzene isomers **1** (444 mg, 1.36 mmol) in dry toluene dropwise at −78 °C under an argon atmosphere. The reaction mixture was allowed to warm to 0 °C, stirred for 1.5 h, quenched by addition of aqueous hydrochloric acid (1 N, 5.0 mL) and extracted with CH_2Cl_2-THF (10:1); the combined organic extracts were dried (Na_2SO_4), filtered, and concentrated. The resulting solid was collected by filtration, washed with dichloromethane and dried under reduced pressure to give the mixture of *trans*- and *cis*-isomers **4** (0.31 g, 84%) as an orange powder which was warmed at 50 °C overnight to afford the pure *trans*-azobenzene isomer **4**.

^{1}H NMR (400 MHz, DMSO) δ (ppm): 2.67 (s, 6H), 4.52 (d, J = 5.8 Hz, 4H), 5.25–5.27 (t, J = 5.7 Hz, 2H), 7.39 (s, 4H), 7.54 (s, 2H). ^{13}C NMR (400 MHz, DMSO) δ (ppm): 17.0, 62.4, 113.4, 129.2, 131.1, 135.7, 141.1, 150.2. IR (solid, ATR): 3320, 2920, 1615, 1500, 1408, 895, 825, 764 cm^{-1}. MS(ESI) m/z: 271 [$(M + H)^+$]. HRMS (ESI): Calcd. for $C_{16}H_{19}N_2O_2^+$: 271.1441 [$(M + H)^+$], Found: 271.1442.

4.1.6. (E)-1,2-bis(5-(Bromomethyl)-2-methylphenyl)diazene **5**

Triphenylphosphine (476 mg, 1.82 mmol) in THF (0.9 mL) was added dropwise to a solution of a mixture of the *trans*- and *cis*-azobenzene isomers **4** (196 mg, 0.73 mmol) and tetrabromomethane (602 mg, 1.82 mmol) in THF (7.0 mL) at room temperature. The reaction mixture was stirred for 3 h, the solvent was removed under reduced pressure and the residue was purified by silica gel column chromatography (hexane/ethyl acetate = 5/1) to afford a mixture of *cis*- and *trans*-isomers **5** (213.1 mg, 0.54 mmol, 74%) as a solid which was warmed at 50 °C overnight to afford the pure *trans*-azobenzene isomer **5**.

^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 2.74 (s, 6H), 4.53 (s, 4H), 7.33–7.35 (d, J = 8.0 Hz, 2H), 7.41–7.43 (dd, J = 2.0, 8.0 Hz, 2H), 7.62 (d, J = 1.6 Hz, 2H). ^{13}C NMR (400 MHz, C_4D_8O) δ (ppm): 16.6, 32.7, 116.2, 131.5, 131.7, 138.2, 151.0. IR (solid, ATR): 656, 744, 867, 1374, 1498, 1609, 2895, 2926 cm^{-1}. MS (ESI) m/z: 394 ($M^{+\bullet}$). HRMS (ESI): Calcd. for $C_{16}H_{17}N_2Br_2^+$ [$(M + H)^+$]: 394.9753, Found: 394.9751.

4.1.7. (E)-3,3′-Diazene-1,2-diyl)bis(4-methylaniline) **6**

Aqueous sodium hydroxide (1 N, 240 mL) was added to a solution of a mixture of the *trans*- and *cis*-azobenzene isomers **1** (4.00 g, 12.2 mmol) in THF (39.3 mL). The reaction mixture was heated under reflux for 19.5 h, cooled to room temperature and acidified by aqueous hydrochloric acid (3 N) to pH ≈ 2 to afford the corresponding acid **16** (3.12 g, 10.4 mmol, 85%) collected by filtration as an orange solid. A solution of the crude acid **16** in dry toluene (229 mL) under argon atmosphere was treated sequentially with triethylamine (5.37 mL, 38.5 mmol), benzyl alcohol (4.0 mL, 38.5 mmol), and diphenylphosphoryl azide (8.31 mL, 38.56 mmol). The resulting reaction mixture was stirred at 80 °C for 24 h, cooled to room temperature, and the solvent removed under reduced pressure. The solid residue was washed with dichloromethane and MeOH to afford **17** (6.45 g, 12.6 mmol, 69%) as an orange solid.

The crude mixture of *trans*- and *cis*-azobenzene isomers **17** (376 mg, 0.74 mmol) in a saturated solution of KOH in ethanol was heated at 100 °C for 24 h. The reaction mixture was cooled to room temperature, then neutralized by addition of saturated aqueous ammonium chloride. The solvents were removed under reduced pressure and the residue extracted with dichloromethane. The combined organic layer was dried (Na_2SO_4). The solvent was removed under reduced pressure and the residue was purified by silica gel column chromatography (CH_2Cl_2/AcOEt = 50/1) to afford a mixture of *trans*- and *cis*-isomers **6** (163 mg, 0.68 mmol, 92%) as an orange solid which was warmed at 50 °C overnight to afford the pure *trans*-azobenzene isomer **6**.

^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 2.61 (s, 6H), 3.64 (brs, 4H, NH_2), 6.73 (dd, J = 2.5, 8.1 Hz, 2H), 6.97 (d, J = 2.5 Hz, 2H), 7.11 (d, J = 8.1 Hz, 2H). ^{13}C NMR (400 MHz, $CDCl_3$) δ (ppm): 16.7, 101.9, 118.3, 128.6, 131.8, 144.8, 151.5. IR (KBr, ATR): 720, 818, 874, 1157, 1292, 1499, 1635, 2362, 2729, 2920, 3024, 3214, 3312, 3403 cm^{-1}. HRMS (EI^+): calcd. for $C_{14}H_{16}N_4^+$ ($M^{+\bullet}$), 240.1375, found, 240.1374.

4.1.8. (E)-3,3'-(Diazene-1,2-diyl)bis(N,4-dimethylaniline) ***7***

A mixture of *cis*- and *trans*-azobenzene **6** was added to a solution of sodium methoxide [prepared by addition of lumps of sodium (139 mg, 6 mmol) to dry methanol (6.8 mL) in an eggplant flask under argon at room temperature]. Then, paraformaldehyde (45.7 mg) was added slowly to the reaction mixture which was stirred at room temperature for 16 h. Sodium borohydride (39.8 mg, 1 mmol) was quickly added to the resulting orange suspension, and a reflux condenser was attached. The mixture was heated under reflux at 70 °C for 6 h. Subsequent addition of aqueous potassium hydroxide (1 N) precipitated a solid which was collected by filtration, washed with water and methanol, and dried under reduced pressure. The residue was purified by silica gel column chromatography (ethyl acetate/hexane = 1/5) to a mixture of *trans*- and *cis*-azobenzene isomers **7** (57 mg, 0.21 mmol, 42%) which on warming at 50 °C overnight, afforded the pure *trans*-isomer **7**.

^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 2.61 (s, 6H, CH_3), 2.86 (s, 6H, CH_3), 6.68 (dd, J = 2.6, 8.2 Hz, 2H, Ar−H), 6.90 (d, J = 2.5 Hz, 2H, Ar−H), 7.14 (d, J = 8.2 Hz, 2H, Ar−H). ^{13}C NMR (400 MHz, $CDCl_3$) δ (ppm): 16.6, 31.0, 99.0, 116.0, 127.3, 131.7, 148.0, 151.6. IR (KBr): 3447, 3295, 3077, 3040, 3012, 2978, 2911, 2801, 2366, 2344, 1609, 1516, 1450,1397, 1256, 1171, 1121, 860, 814 cm^{-1}. MS (ESI) m/z: 269 [(M + H)$^+$]. HRMS (ESI) m/z: Calcd. for $C_{16}H_{21}N_4^+$ [(M + H)$^+$]: 269.1766, Found: 269.1761.

4.1.9. (E)-3′,3‴-(Diazene-1,2-diyl)bis(4′-methyl-[1,1′-biphenyl]-2-amine) ***8***

A biphasic reaction mixture of Oxone® (13.25 g, 21.5 mmol) in water (130 mL) was stirred with a solution of 5-bromo-2-methylaniline **18** (2.0 g, 10.75 mmol) in dichloromethane (32 mL) at room temperature for 3.5 h. The organic layer was then separated and the aqueous layer was extracted with CH_2Cl_2. The combined organic layers were washed with 1 N HCl, sat. aq. $NaHCO_3$, water, and brine. Then, the organic phase was dried over Na_2SO_4, filtered, and evaporated under reduced pressure to give the nitroso compound **19** which was used in the next step without further purification.

5-Bromo-2-methylaniline **18** (2.2 g, 11.1 mmol) was added to the solution of the crude **19** in acetic acid (50 mL) and the reaction mixture stirred at room temperature for 25 h. The precipitate formed was collected by filtration, washed with water and 1 N AcOH (132 mL), and dried under reduced pressure to afford 5,5′-dibromo-2,2′-dimethylazobenzene **20** (2.2 g, 53%) as an orange solid.

Argon was bubbled through a biphasic mixture of toluene (26 mL), ethanol (2 mL), aqueous sodium carbonate (2 M, 1 mL) for 40 min in an ice bath. The mixture of *trans*- and *cis*-azobenzene isomers **20** (200 mg, 0.54 mmol), (2-aminophenyl)boronic acid **21** (222 mg, 1.62 mmol), and $Pd(PPh_3)_4$ (37 mg, 6 mol%) were added to another two-necked flask, dried, and placed under argon atmosphere. The above Na_2CO_3 solution was added to the flask, and the reaction mixture was heated under reflux at 100 °C for 2 h. The reaction mixture was then extracted with ethyl acetate; the solvent was removed under reduced pressure to produce crude **8** which was suspended in methanol and filtered. The filter was washed with CH_2Cl_2 to provide red-black solid and orange filtrate. The solvent of the filtrate was removed under reduced pressure. The residue was passed through a short pad of silica gel prepared with dichloromethane to obtain **8** as mixture of *trans*- and *cis*-azobenzene isomers (189 mg, 0.48 mmol, 89%) which when warmed at 50 °C overnight afforded the pure *trans*-azobenzene isomer **8**.

^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 2.74 (s, 6H, CH_3), 3.79 (s, 4H, NH_2), 6.78−6.86 (m, 4H, Ar−H), 7.14−7.20 (m, 4H, Ar−H), 7.40 (d, J = 7.8 Hz, 2H, Ar−H), 7.47 (dd, J = 1.8, 7.8 Hz, 2H, Ar−H), 7.47 (d, J = 1.8 Hz, 2H, Ar−H). [cis] 2.44 (s, 6H, CH_3), 3.19 (s, 4H, NH_2), 6.32 (d, J = 1.5 Hz, 2H, Ar−H), 6.60 (d, J = 8.1 Hz, 2H, Ar−H), 6.70 (t, J = 7.8 Hz, 2H, Ar−H), 6.81−6.83 (m, 2H, Ar−H), 7.07 (t, J = 7.8 Hz, 2H, Ar−H), 7.14−7.20 (m, 2H, Ar−H), 7.31 (d, J = 7.8 Hz, 2H, Ar−H). ^{13}C NMR (400 MHz, $CDCl_3$) δ (ppm): 17.5, 115.6, 116.4, 118.7, 127.0, 128.6, 130.5, 131.5, 131.8, 137.1, 137.7, 143.6, 151.3. IR (solid, KBr): 754, 823, 1172, 1305, 1371, 1453, 1485, 1619, 2353, 1485, 1619, 2359, 2916, 3012, 3354, 3441 cm^{-1}. MS (ESI) m/z: 393 [(M+1)$^+$]. HRMS (ESI) m/z: Calcd. for $C_{26}H_{25}N_4^+$: 393.20707, Found: 393.20737.

4.1.10. (E)-1-(4-Methyl-3-((2-methyl-5-(naphthalen-1-yl)phenyl) diazenyl)phenyl)naphthalen-2-ol ***9***

Argon was bubbled through a solution of Na_2CO_3 (123 mg) in water (0.58 mL) for 10 min. Then, this solution was added to a solution of **6** (a mixture of *trans*- and *cis*-azobenzene isomers) (100 mg, 0.27 mmol), 2-methoxy-1-naphthaleneboronic acid **22** (136 mg, 0.68 mmol), and $Pd(PPh_3)_4$ (11.1 mg, 0.0096 mmol) in degassed DME (1.2 mL) under an argon atmosphere. The reaction mixture was refluxed for 24 h, and then the solvent was evaporated under reduced pressure. Water was added to the residue, and the reaction mixture extracted with dichloromethane; the extract was dried (Na_2SO_4), and the solvent removed to give a residue which was purified by silica gel column chromatography (ethyl acetate/hexane = 1/10) to provide **23** as a mixture of *trans*- and *cis*-azobenzene isomers (120 mg, 85%), an orange solid.

To a solution of **23** (100 mg, 0.19 mmol) in CH_2Cl_2 (0.76 mL) was added a 17% boron tribromide solution in dichloromethane (0.57 mL) at 0 °C. The solution was gradually warmed to room temperature and then stirred overnight. Then water and sat. aq. $NaHCO_3$ were added to quench the reaction. The reaction mixture was extracted with dichloromethane, the combined organic layers were dried (Na_2SO_4), filtered, and the solvent removed. The residue was purified by silica gel column chromatography (hexane/EtOAc = 5/1) to afford **9** as a mixture of *trans*- and *cis*-azobenzene isomers (86 mg, 92%) which on being warmed at 50 °C overnight to afforded the pure *trans*-azobenzene isomer **9**.

^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 2.76 (s, 6H), 5.17 (d, J = 2.3 Hz, 2H), 7.28 (d, J = 8.9 Hz, 2H), 7.33−7.38 (m, 4H), 7.41−7.48 (m, 4H), 7.54 (d, J = 7.7 Hz, 2H), 7.2 (d, J = 1.5 Hz, 2H), 7.83 (d, J = 9.1 Hz, 2H). ^{13}C NMR (400 MHz, $CDCl_3$) δ (ppm): 17.6, 117.4, 118.4, 120.4, 123.4, 124.6, 126.6, 128.1, 128.9, 129.6, 132.3, 132.7, 133.283 133.7, 138.7, 150.3, 151.6. IR (neat): 3536, 3364, 3057, 2359, 1938, 1622, 1598, 1496, 1465, 1434, 1390, 1334, 1271, 1227, 1214, 1196, 1146, 836, 816, 752, 743 cm^{-1}. MS (ESI) m/z: 495 [(M + H)$^+$]; HRMS (ESI): Calcd. for $C_{34}H_{27}N_2O_2^+$: 495.2067, Found: 495.2064.

4.1.11. (E)-1-(3-((5-(Diphenylphosphaneyl)-2-methylphenyl) diazenyl)-4-methylphenyl)naphthalen-2-ol ***10***

5-Bromo-2-methylaniline **18** (1.86 g, 10.0 mmol) was converted to the corresponding nitroso compound **19** as described is section 4.1.9 above.

5-Iodo-2-methylaniline **24** (1.66 g, 7.1 mmol) was added to the solution of the crude nitroso product **19** in acetic acid-EtOAc (2:1, 102 mL) at room temperature under argon. After stirring overnight, a precipitate formed in the reaction mixture was collected by filtration, washed with hexane, and dried under reduced pressure to afford 5-bromo-5′-iodo-2,2′-dimethylazobenzene **25** (2.14 g, 73%) as an orange solid.

A solution of the iodoazo compound **25** (166 mg, 0.4 mmol), diphenylphosphine (82 μL, 0.48 mmol), diphenylphosphine oxide (64.6 mg, 0.32 mmol), palladium diacetate (9.06 mg, 0.04 mmol), triethylamine (89 μL, 0.64 mmol) and acetonitrile (7.2 mL) in a sealed test tube was stirred for 12.5 h at 80 °C in an argon atmosphere. The reaction mixture was evaporated to give crude product **26** which was purified by silica gel column chromatography (hexane/EtOAc = 1/2) to afford the azophosphine oxide **26** as a mixture

of *trans*- and *cis*-azobenzene isomers (910 mg, 77%).

A solution of Na_2CO_3 (123.4 mg) in water (0.58 mL) prepared as in section 4.1.9 was added to a solution of the azo diphenylphosphine oxide **26** (1.47 g, 3.0 mmol), 2-methoxy-1-naphthaleneboronic acid **22** (606 mg, 3 mmol), and $Pd(PPh_3)_4$ (124 mg, 0.108 mmol, 3.6 mol%) in degassed DME (1.2 mL) under an argon atmosphere. The reaction mixture was heated under reflux for 24 h, and then the solvent was removed under reduced pressure. Water was added to the residue, and the reaction mixture then extracted with dichloromethane, dried (Na_2SO_4), filtered, and the solvent removed. The residue was purified by silica gel column chromatography (ethyl acetate/hexane = 1/2) to provide a pure mixture of *trans*- and *cis*-azobenzene isomers **27** (1.85 g, quant.) as an orange solid.

A boron tribromide solution in dichloromethane (1.0 M, 1.5 mL, 1.5 mmol) was added to the mixture of *trans*- and *cis*-azobenzenes **27** (566 mg, 1.0 mmol) in dichloromethane (4.0 mL) at 0 °C under an argon atmosphere. The reaction mixture was gradually warmed to room temperature, stirred overnight, quenched with water (4 mL) and sat. aq. $NaHCO_3$ and then extracted with dichloromethane. The combined organic extracts were dried (Na_2SO_4), filtered, and concentrated. The residue was purified by silica gel column chromatography (hexane/EtOAc = 1/3) to afford **28** as a mixture of *trans*- and *cis*-azobenzene isomers (388 mg, 70%).

A solution of the *trans*- and *cis*-azobenzene mixture **28** (276 mg, 0.5 mmol, 1.0 equiv.), diphenyl phosphate (10 mg, 0.04 mmol, 0.08 equiv.), and trichlorosilane (320 μL, 2 mmol, 4.0 equiv.) in 1,4-dioxane (2 mL) in a sealed test tube under argon was stirred and heated at 110 °C for 48 h. Then, methanolic KOH (5.0 mL) was added slowly at 0 °C to the reaction mixture which was stirred vigorously for 3 h at room temperature; water (3 mL) was added and the mixture was extracted by ethyl acetate. The organic phase was washed by aqueous hydrochloric acid (1 N, 5 mL) and sat. aq. $NaHCO_3$ (5 mL), dried (Na_2SO_4), filtered, and the solvent removed under reduced pressure. The residue was purified by silica gel column chromatography (hexane/CH_2Cl_2 = 1/1) to afford **10** as a mixture of *trans*- and *cis*-azobenzene isomers (66.25 mg, 24%) as an orange solid which was warmed at 50 °C overnight to produce the pure *trans*-azobenzene isomer **10**.

^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 2.66 (s, 3H), 2.68 (s, 3H), 7.28–7.35 (m, 3H), 7.40–7.58 (m, 10H), 7.65–7.73 (m, 6H), 7.79–7.84 (m, 3H). ^{13}C NMR (400 MHz, $CDCl_3$) δ (ppm): 17.4, 17.8, 117.5, 118.2, 120.2, 120.3, 123.4, 124.5, 126.6, 128.1, 128.5, 128.6, 128.9, 129.6, 132.0, 132.1, 132.2, 132.68, 134.0, 150.3, 151.2. IR (neat): 3054, 1624, 1512, 1496, 1435, 1345, 1287, 1174, 1119, 1106, 821, 748, 723, 705, 691, 546, 523 cm^{-1}. MS (ESI) *m/z*: 537.20 [$(M + H)^+$]. HRMS (ESI): Calcd. for $C_{36}H_{30}N_2OP^+$ [$(M + H)^+$]: 537.20914, Found: 537.20903.

*4.1.12. (E)-1-(3,5-bis(trifluoromethyl)phenyl)-3-(3-((5-((4-(dimethylamino)pyridin-2-yl)amino)-2-methylphenyl)diazenyl)-4-methylphenyl)thiourea **11***

A solution of **6** (120 mg, 0.5 mmol), 2-iodo-4-dimethylaminopyridine **29** (124.2 mg, 0.5 mmol), Pd_2dba_3 (49.8 mg, 0.05 mmol), 1,3-bis(diphenylphosphino)propane (37.1 mg, 0.09 mmol) and sodium *tert*-butoxide (96.1 mg, 1.0 mmol) in degassed toluene was heated at 105 °C for 2 h under argon. The reaction mixture was cooled to room temperature and was filtered through a pad of celite which was washed with CH_2Cl_2. The filtrate was washed with water, dried (Na_2SO_4), and evaporated under reduced pressure to give a residue which was purified by silica gel column chromatography ($CHCl_3$/MeOH = 20/1) to give DMAP azo analogue **30** as a mixture of *trans*- and *cis*-azobenzene isomers (83.7 mg, 47%) as a red solid.

3,5-*bis*(Trifluoromethyl)phenyl isothiocyanate **31** (134 μL, 0.73 mmol) was added dropwise to a solution of **30** (240 mg, 0.67 mmol) in dry CH_2Cl_2 (1.3 mL) at 0 °C under an argon atmosphere. The solution was gradually warmed to room temperature and stirred for 5 h. The solvent of the reaction mixture was evaporated to give a residue which was washed with $CHCl_3$ to provide **11** as a mixture of *trans*- and *cis*-azobenzene isomers (237 mg, 50%), a yellow solid which was warmed at 50 °C overnight to afford the pure *trans*-azobenzene isomer **11**.

^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 2.64 (s, 3H), 2.71 (s, 3H), 2.96 (s, 6H), 6.07 (d, J = 2.2 Hz, 1 H), 6.15 (dd, J = 2.2, 6.1 Hz, 1 H), 7.33 (m, 3 H), 7.43 (d, J = 8.1 Hz, 1 H), 7.54 (d, J = 2.3 Hz, 1H), 7.60 (d, J = 2.3 Hz, 1H), 7.68 (s, 1H), 7.85 (d, J = 6.1 Hz, 1H), 8.02 (s, 1H). ^{13}C NMR (400 MHz, DMSO) δ (ppm): 16.3, 16.6, 91.0, 100.9, 104.1, 110.6, 116.8, 121.6, 121.9, 123.3, 124.6, 126.6, 129.1, 129.54, 129.9, 130.2, 130.5, 131.2, 131.5, 134.4, 137.3, 141.1, 141.9, 147.0, 150.2, 150.2, 155.4, 156.6, 179.9. IR (solid, ATR): 681, 884, 986, 1136, 1177, 1278, 1385, 1500, 1667, 2359, 2819, 2866, 2924, 3024, 3298, 3425 cm^{-1}. HRMS (ESI): Calcd. for $C_{30}H_{28}N_7F_6S^+$ [$(M + H)^+$]: 632.20256, Found: 632.20243.

*4.1.13. (E)-1-(3,5-bisTtrifluoromethyl)phenyl)-3-(3-((5-((4-(dimethylamino)pyridin-2-yl)amino)-2-methylphenyl)diazenyl)-4-methylphenyl)urea **12***

1-Isocyanato-3,5-bis(trifluoromethyl)benzene **32** (53.0 μL, 0.31 mmol) was added dropwise to a solution of the DMAP *trans*-*cis*- isomers **30** (100 mg, 0.28 mmol) in dry CH_2Cl_2 (0.54 mL) at 0 °C under an argon atmosphere. The solution was gradually warmed to room temperature and was stirred for 24 h. Then, the solvent of the reaction mixture was evaporated, and the residue was purified by silica gel column chromatography ($CHCl_3$/MeOH/triethylamine = 10/1/a few drops) to provide **12** as a mixture of *trans*- and *cis*-azobenzene isomers (71.2 mg, 0.12 mmol, 42%) as a yellow solid which was warmed at 50 °C overnight to afford the pure *trans*-azobenzene isomer **12**.

^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 2.43 (s, 3H), 2.45 (s, 3H), 3.01 (s, 6H), 5.98 (s, 1H), 6.20 (dd, J = 2.17, 6.37 Hz, 1H), 7.06 (s, 2H), 7.12 (d, J = 8.20 Hz, 1H), 7.30 (d, J = 6.70 Hz, 1H), 7.45 (s, 2H), 7.81 (d, J = 6.30 Hz, 1H), 7.85 (s, 2H). ^{13}C NMR (400 MHz, DMSO) δ (ppm): 16.5, 90.9, 101.0, 104.7, 105.7, 114.3, 118.0, 121.6, 121.9, 121.9, 124.7, 127.4, 129.1, 130.2, 130.5, 130.8, 131.2, 131.6, 131.6, 137.6, 140.8, 141.8, 146.4, 150.3, 150.4, 152.5, 155.5, 156.2. IR (solid, ATR): 893, 954, 987, 1011, 1064, 1128, 1176, 1281, 1386, 1448, 1472, 1499, 1566, 1604, 1643, 1714, 2324, 2351, 2872, 2925, 3273 cm^{-1}. HRMS (ESI): Calcd. for $C_{34}H_{27}N_2O_2^+$ [$(M + H)^+$]: 616.22540, Found: 616.22509.

*4.1.14. (E)-1-(3,5-bis(Trifluoromethyl)phenyl)-3-(4-methyl-3-((2-methyl-5-(pyridin-2-ylamino)phenyl)diazenyl)phenyl)thiourea **13***

A solution of 6 (200.0 mg, 0.83 mmol), 2-iodo-pyridine **33** (88 μL, 0.83 mmol), Pd_2dba_3 (73.2 mg, 0.08 mmol, 10 mol%), 1,3-bis(diphenylphosphino)propane (57.8 mg, 0.14 mmol), and sodium *tert*-butoxide (160 mg, 1.66 mmol) in degassed dry toluene (6.6 mL) was heated at 105 °C for 2 h under an argon atmosphere. Then, the reaction mixture was cooled to room temperature and filtered through a pad of celite which was rinsed with dichloromethane. The filtrate was washed with water and dried (Na_2SO_4), and evaporated under reduced pressure to give a residue which was purified by silica gel column chromatography (MeOH/$CHCl_3$ = 1/20) to afford **34** (82.0 mg, 0.38 mmol, 46%).

3,5-*bis*(Trifluoromethyl)phenyl isothiocyanate **31** (232 μL, 1.27 mmol) was added dropwise to a solution of **34** as a mixture of *trans*- and *cis*-azobenzene isomers (405 mg, 0.67 mmol) in dry dichloromethane (1.3 mL) at 0 °C under argon. The solution was gradually warmed to room temperature and was stirred for 5 h. Then, the solvent of the reaction mixture was evaporated, and the residue was washed with $CHCl_3$ to provide **13** as a mixture of *trans*- and *cis*-azobenzene isomers (432 mg, 0.73 mmol, 57%) as a yellow

solid which was warmed at 50 °C overnight to afford the pure *trans*-azobenzene isomer.

^{1}H NMR (400 MHz, DMSO) δ (ppm): 2.58 (s, 3H), 2.71 (s, 3H), 6.75 (t, J = 5.97 Hz, 1H), 6.83 (d, J = 8.40 Hz, 1H), 7.31 (d, J = 8.41 Hz, 1H), 7.44 (d, J = 8.28 Hz, 1H), 7.57 (m, 2H), 7.72 (s, 1H), 7.81 (m, 2H), 8.03 (d, J = 1.89 Hz, 1H), 8.15 (d, J = 3.74 Hz, 1H), 8.25 (s, 2H), 9.15 (s, 1H), 10.31 (brs, 2H). ^{13}C NMR (400 MHz, DMSO) δ (ppm): 16.8, 17.1, 104.9, 111.1, 111.3, 114.8, 117.3, 122.1, 122.4, 123.8, 125.1, 127.2, 130.3, 130.7, 131.8, 132.0, 135.0, 137.7, 137.9, 140.9, 142.4, 147.6, 150.7, 156.3, 180.4. IR (solid, ATR): 702, 890, 1126, 1278, 1385, 1530, 1602, 2322, 2351, 2372, 2972, 3224 cm^{-1}. HRMS (ESI): Calcd for $C_{28}H_{22}N_6F_6S^+$ [(M + H)$^+$]: 589.16036, Found: 589.16024.

4.1.15. (E)-1-(3,5-bis(Trifluoromethyl)phenyl)-3-(4-methyl-3-((2-methyl-5-((4-(pyrrolidin-1-yl)pyridin-2-yl)amino)phenyl)diazenyl)phenyl)thiourea **14**

A solution of **6** (500 mg, 2.08 mmol), 2-iodo-4-(pyrrolidin-1-yl) pyridine **35** (571 mg, 2.08 mmol), Pd_2dba_3 (190 mg, 0.21 mmol), 1,3-bis(diphenylphosphino)propane (154 mg, 0.37 mmol), and sodium *tert*-butoxide (400 mg, 4.16 mmol) in degassed dry toluene (27 mL) was heated at 105 °C for 15 h under an argon atmosphere. Then, the reaction mixture was cooled to room temperature and filtered through a pad of celite which was rinsed with dichloromethane. The filtrate was washed with water and dried (Na_2SO_4) and the solvent evaporated under reduced pressure to give a residue which was purified by silica gel column chromatography (MeOH/AcOEt = 1/4) to afford the pyrrolidine azo analogue **36** (834 mg, 0.88 mmol, 42%).

3,5-*bis*(Trifluoromethyl)phenyl isothiocyanate **31** (73.1 μL, 0.39 mmol) was added dropwise to a solution of a mixture of *trans*- and *cis*-azobenzene isomers of **36** (150 mg, 0.39 mmol) in dry CH_2Cl_2 (3.9 mL) at 0 °C under an argon atmosphere. The solution was gradually warmed to room temperature and was stirred for 5 h. Then, removal of the solvent gave a residue which was purified by silica gel column chromatography (MeOH/$CHCl_3$ = 1/6) to provide **14** as a mixture of *trans*- and *cis*-azobenzene isomers (101 mg, 0.15 mmol, 40%) as an orange solid which was warmed at 50 °C overnight to afford the pure *trans*-azobenzene isomer **14**.

^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 1.99 (m, 4H, CH_2), 2.64 (s, 3H, CH_3), 2.71 (s, 3H, CH_3), 3.28 (m, 4H, CH_2), 5.97 (d, J = 2.1 Hz, 1H, Ar−H), 6.04 (dd, J = 2.1, 6.0 Hz, 1H, Ar−H), 7.08 (bs, 1H, NH), 7.28−7.36 (m, 3H, Ar−H), 7.44 (d, J = 8.0 Hz, 1H, Ar−H), 7.55 (d, J = 2.2 Hz, 1H, Ar−H), 7.59 (d, J = 2.2 Hz, 1H, Ar−H), 7.68 (s, 1H, Ar−H), 7.86 (d, J = 6.0 Hz, 1H, Ar−H), 8.02 (s, 2H, Ar−H). ^{13}C NMR (400 MHz, DMSO) δ (ppm): 16.3, 16.5, 46.6, 90.9, 101.3, 104.0, 110.6, 121.6, 121.9, 123.3, 124.6, 129.0, 129.9, 130.2, 131.2, 141.2, 147.0, 150.0, 152.6, 156.4. IR (solid, ATR): 1179, 1209, 1232, 1274, 1330, 1354, 1387, 1484, 1506, 1529, 1557, 1607, 1641, 2325, 2351, 2373, 2840, 2874, 2974, 3088, 3296 cm^{-1}. HRMS (EI): Calcd. for $C_{32}H_{30}N_7F_6S^+$ [(M + H)$^+$]: 658.21821, Found: 658.21751.

4.2. Biological assay

4.2.1. Cell culture

Human pancreatic cancer cell line PANC-1 was provided by Dr. Yukiko Kurashima (Hokuriku University). Human normal fibroblast cell line GM05659 was obtained from Coriell Institute for Medical Research (Camden, NJ, USA). Cells were cultured in the following media: Dulbecco's modified Eagle's medium (DMEM; FUJIFILM Wako Pure Chemical, Osaka, Japan) supplemented with 10% heat-inactivated bovine serum (BS; Thermo Fischer Scientific, MA, USA), non-essential amino acids (FUJIFILM Wako Pure Chemical, Osaka, Japan), and penicillin-streptomycin (100 U/mL and 100 μg/mL, respectively; Thermo Fischer Scientific, MA, USA) for PANC-1 cells; Eagle's minimum essential medium (EMEM; FUJIFILM Wako Pure Chemical, Osaka, Japan) supplemented with 15% BS and penicillin-streptomycin (100 U/mL and 100 μg/mL, respectively) for GM05659 cells. The cells were cultured at 37 °C under 5% CO_2. In cases of experiments under nutrient-deprived conditions, the cells were cultured in nutrient-deprived medium (NDM), which is inclusive of electrolytes and vitamins but exclusive of glucose, amino acids, and serum. NDM was prepared as described previously [6].

4.2.2. Cell viability assay

Cell viability was assessed by MTT assay. All test samples were dissolved in dimethyl sulfoxide (DMSO). Cells were seeded at 1.0×10^4 (PANC-1) or 5.0×10^3 (GM05659) cells/well in a 96-well plate and precultured for 24 h. After preculture, the cells were washed with phosphate-buffered saline (PBS) and exposed to serial dilutions of test samples under either normal conditions (DMEM) or nutrient-deprived conditions (NDM). The final concentration of DMSO was 0.1%. After 24 or 72 h of incubation, 20 μL of MTT solution (2.0 mg/mL) was added to wells, and cells were incubated at 37 °C for 4 h. Then, the culture medium was removed and crystals of formazan were dissolved with DMSO (200 μL/well). Absorbance at 540 nm was measured by using the FilterMax F5 microplate reader (Molecular Devices, San Jose, CA, USA). Results were expressed as percentage of the values of control cells (mean ± SEM).

4.2.3. Cell cycle analysis by using flow cytometry

Cell cycle of PANC-1 cells was determined by flow cytometric analysis after staining with propidium iodide (PI). PANC-1 cells were seeded at 2.0×10^5 cells/well in a 6-well plate and precultured for 24 h. The cells were washed with PBS and exposed to test samples under either normal conditions (DMEM) or nutrient-deprived conditions (NDM). After 24 h of incubation, the cells were trypsinized and washed twice with PBS. The cells were fixed in ice-cold 70% ethanol and placed overnight at −20 °C. The fixed cells were washed with PBS containing 2% BS, and then 0.25 mg/mL RNAse (Nacalai Tesque Inc, Kyoto, Japan) was added and incubated at 37 °C for 15 min. The cells were stained with PI (FUJIFILM Wako Pure Chemical, Osaka, Japan) solution at a final concentration of 50 μg/mL and finally analyzed by using the FACSCelesta cytometer and FACSDiva software, ver. 9.0 (Becton Dickinson, Franklin Lakes, NJ).

Declaration of competing interest

None of the authors have any conflict of interest arising from this work.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.tet.2021.132077.

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