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Solid Phase Synthesis and Biological Activity of Tuftsin Conjugates

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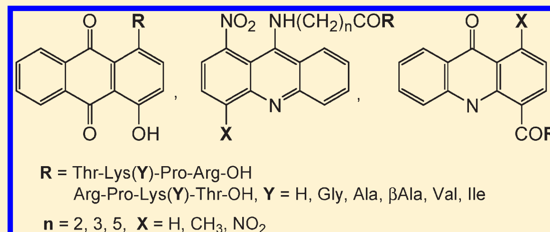
S Supporting Information

ABSTRACT: New tuftsin/retro-tuftsin conjugates were designed and synthesized using a classical fluorenylmethoxycarbonyl (Fmoc) solid phase procedure. All the peptide conjugates were divided into three series: 1,4-dihydroxyanthraquinone (type A), 1-nitroacridine (type B), and 4-carboxyacridone (type C) derivatives. In type A conjugates, the N-terminal group of the peptide chain is directly connected to the anthraquinone ring at C1 (Scheme 1), whereas types B and C conjugates possess an amide bond formed between the carboxyl group of heterocyclic molecule and the N-termini of the tuftsin chain. The in vitro cytotoxic activity of the tuftsin conjugates and their precursors using two human tumor cell lines (lung adenocarcinoma (A549) and myeloblastic leukemia (HL-60)) was investigated. The analogues from groups A and C exhibited low cytotoxic activity, whereas several compounds of type B showed a potent and selective cytotoxic activity against tested tumor cell lines. None of the examined tuftsin conjugates demonstrated any significant effect on the catalytic activity of types I and II DNA topoisomerases.



INTRODUCTION

Many acridine derivatives possess antimicrobial, antiviral, and anticancer properties.[1,2] These compounds are also known as biological fluorescent probes. Acridine derivatives are DNA-binding agents but also interact with other biological targets such as types I and II DNA topoisomerases, telomerase, polymerase, and protein kinases.[1,3] One of the acridine derivatives that showed potent anticancer properties was ledakrin (nitracrine). This compound was introduced into the clinic as an antitumor drug with unique activity against many solid tumors.[4] Unfortunately, ledakrin produced intense nausea and vomiting in cancer patients and was eventually withdrawn from clinical practice. Recently, new 4-methyl analogues of ledakrin and their analogues were synthesized and showed promising antitumor activity and much lower general toxicity.[5]

The second group of compounds, which has found clinical applications in the treatment of leukemia or solid tumors, is anthracycline antibiotics. However, these drugs, like many other chemotherapeutics, also show toxic effects such as high cardiotoxicity that limit their application as antitumor drugs. Search for novel anthracycline analogues with improved pharmaceutical properties led to the development of mitoxantrone, a second generation synthetic drug used in cancer therapy. However, similar to anthracyclines, mitoxantrone demonstrates cardiotoxic properties as a consequence of the generation of reactive oxygen species in living cells.[6−8]

It is well-known that an ideal chemotherapeutic agent should selectively target neoplastic cells with minimal adverse effects on healthy cells. One of the approaches that can lead to increased drug specificity is the design of peptide-based conjugates, where the peptidic component serves to selectively target tumor cells. Many peptide−drug derivatives showed increased specificity toward tumor cells and low general toxicity,[9−12] in part through their binding to specific receptors that are present on the cell surface.[9,13] These include peptides combined with anthraquinone and acridine/acridone derivatives that showed encouraging pharmacological properties.[1,3]

In this study, we applied tuftsin analogues as a peptidyl carrier of cytotoxic molecules. Tuftsin is a tetrapeptide (TKPR) that is liberated from the Fc domain of the heavy chain of immunoglobulin G (IgG) by two enzymes, leukokininase and spleen tuftsin endocarboxypeptidase. This peptide activates several components of the immune system, including granulocytes and macrophages.[14] Tuftsin has been successfully investigated as a coadministration agent with different antibiotics in the treatment of opportunistic infections caused by bacteria, fungi, and viruses, but it also showed antineoplastic properties.[15−17] Since tuftsin is prone to a rapid enzymatic degradation in the blood plasma,[18,19] we designed peptide conjugates containing tuftsin analogues modified at the ε-amino group of lysine to increase their blood plasma stability. We hypothesized that covalent combination of tuftsin analogues and anthraquinone or acridine/acridone derivatives will allow us to obtain conjugates with anticancer activity and improved selectivity toward tumor cells. In this paper, we present the solid-phase synthesis of new tuftsin conjugates with anthraquinone and acridine/acridone derivatives and evaluation of their cytotoxic activity as well as biological effects induced by these compounds in tumor cells.

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Scheme 1. Synthesis of Tuftsin−Anthraquinone Conjugates, 4

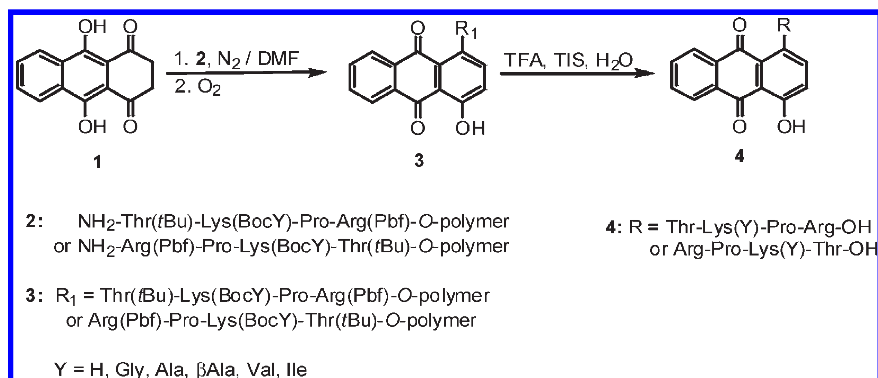

Table 1. Tuftsin−Anthraquinone Conjugates 4a−k

compd	R	Y	HPLC t_R [min]	yield [%]
4a	Thr-Lys(Y)-Pro-Arg-OH	Ala	18.48	34
4b	Thr-Lys(Y)-Pro-Arg-OH	*β*Ala	18.49	31
4c	Thr-Lys(Y)-Pro-Arg-OH	Val	18.67	33
4d	Thr-Lys(Y)-Pro-Arg-OH	Gly	18.51	33
4e	Thr-Lys(Y)-Pro-Arg-OH	Ile	18.63	42
4f	Arg-Pro-Lys(Y)-Thr-OH	H	18.62	45
4g	Arg-Pro-Lys(Y)-Thr-OH	Ala	18.79	38
4h	Arg-Pro-Lys(Y)-Thr-OH	*β*Ala	18.58	41
4i	Arg-Pro-Lys(Y)-Thr-OH	Val	18.65	35
4j	Arg-Pro-Lys(Y)-Thr-OH	Gly	18.73	34
4k	Arg-Pro-Lys(Y)-Thr-OH	Ile	18.63	40

RESULTS AND DISCUSSION

Chemistry. *Solid Phase Synthesis of the Tuftsin−Resin.* The tuftsin analogues were synthesized on polymeric support using the Fmoc chemistry. The first amino acid was attached directly to resin via the carboxylic group. The method of the peptide chain elongation was based on a two-step procedure involving deprotection and coupling. The removal of the α-amino protecting group was achieved by treatment with piperidine. The coupling reaction was performed with *N,N'*-diisopropylocarbodiimide (DIC) and *N*-hydroxybenzotriazole (HOBt) in a solvent mixture. Modification of the tuftsin chain was based on the introduction of the additional amino acid (Gly, Val, Ile, Ala, or *β*-Ala) into the peptide chain at the *ε*-amino group of lysine residue. To build a tuftsin-containing isopeptide bond, the routinely used Fmoc-Lys-*tert*-butoxylcarbonyl-OH was replaced by Fmoc-Lys-4-methyltrityl-OH. The selective removal of the 4-methyltrityl (Mtt) group was induced by mild acidolysis.[20] The free *ε*-amino group of lysine was attached to the appropriate Boc-amino acid using *O*-benzotriazol-1-yl-*N,N,N',N'*-tetramethyluronium tetrafluoroborate (TBTU) as a coupling reagent, HOBt as an additive to suppress racemization, and *N,N*-diisopropylethylamine (DIPEA) as a base. The strategy for the modification of tuftsin and retro-tuftsin was reported in detail previously.[18] The final deprotection step was performed with piperidine to obtain the free N-terminal groups of peptides. Next, the tuftsinyl resin was applied to prepare the peptide−anthraquinone, peptide−acridine, and peptide−acridone conjugates.

Synthesis of the Conjugates of Tuftsin or Retro-Tuftsin with 1,4-Dihydroxyanthraquinone (Type A). The solid-phase synthesis of new tuftsin conjugates was carried out according to Scheme 1. The condensation between leuco-1,4-dihydroxy-9,10-anthraquinone (**1**) and the N-termini of the peptide−resin (**2**) was achieved during reaction in *N,N*-dimethylformamide (DMF) at 120 °C under N_2 for 24 h. A 3-fold excess of leuco-anthraquinone was used in the reaction. Then peptidyl-anthraquinone-resin was oxidized by air oxygen at room temperature.[21,22] To cleave the product from resin with simultaneous removal of all the side chain protecting groups, dried peptidyl-anthraquinone-resin was treated with a mixture of trifluoroacetic acid (TFA), triisopropylsilane (TIS), and water. The tuftsin conjugates were purified by solid-phase extraction (SPE) and preparative HPLC (Table 1).

The leuco compound was synthesized using both protecting and good leaving groups in the quinizarin moiety. Compound **1** is more liable to create a covalent bond between the N-terminal group of the peptide and the anthraquinone unit.[23] This solid phase procedure is recommended for obtaining monosubstituted anthraquinones.

Synthesis of Tuftsin and Retro-Tuftsin Conjugates with 1-Nitroacridine/4-Carboxyacridone Derivatives (Types B and C). The synthesis of types B and C tuftsin conjugates was carried out according to Scheme 2. Tuftsin analogue on a solid support was bound to the acridine molecule via flexible linkers. The carboxylic group of linker was attached to the N-terminal group of peptide−resin using a standard method for amide bond formation (type B). Peptide on the polymeric support was linked to acridone molecules via a 4-carboxyamide bond (type C).[24−26] The coupling reactions were performed with a 2-fold molar excess of acridine or acridone and a 4-fold molar excess of activating reagents (TBTU/HOBt) in the presence of DIPEA in dry DMF at room temperature for 48 h. Unreacted heterocyclic compounds were easily removed by filtration. Then dried peptidyl-chromophore-resin was treated with TFA to liberate the final product from the resin with simultaneous removal of all protecting groups in the side chain. Unreacted peptide and peptide−acridine or peptide−acridone were the only products. The SPPS method for amide bond formation produced crude compounds with good purity. The final products (both types) were further purified by SPE and preparative RP-HPLC (Table 2).

Biological Assays. First, we evaluated the cytotoxic activity of tuftsin conjugates against two types of human tumors: lung

Scheme 2. Synthesis of Tuftsin−Acridine/Acridone Conjugates

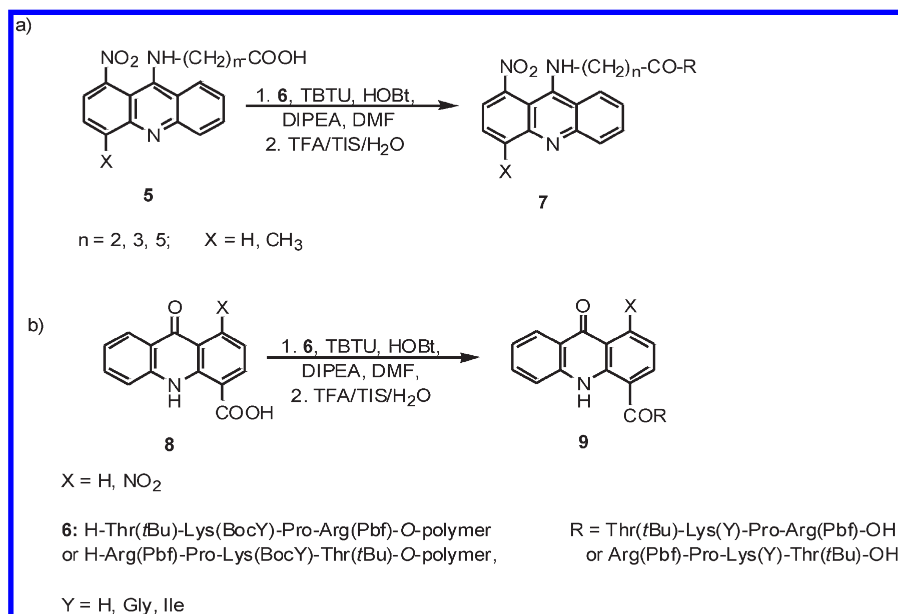

Table 2. Tuftsin−Acridine Conjugates (7a−h) and Tuftsin−Acridone Conjugates (9a−i)

compd	*n*	X	R	Y	HPLC t_R [min]	yield [%]
7a	3	CH_3	Thr-Lys(Y)-Pro-Arg-OH	Gly	23.49	38
7b	2	CH_3	Thr-Lys(Y)-Pro-Arg-OH	Gly	21.51	50
7c	2	H	Thr-Lys(Y)-Pro-Arg-OH	Gly	21.59	40
7d	5	CH_3	Thr-Lys(Y)-Pro-Arg-OH	Ile	22.39	48
7e	5	CH_3	Thr-Lys(Y)-Pro-Arg-OH	H	22.15	43
7f	2	H	Arg-Pro-Lys(Y)-Thr-OH	Gly	21.70	52
7g	5	CH_3	Arg-Pro-Lys(Y)-Thr-OH	Ile	23.10	42
7h	5	CH_3	Arg-Pro-Lys(Y)-Thr-OH	H	23.01	47
9a		H	Thr-Lys(Y)-Pro-Arg-OH	H	18.37	43
9b		H	Thr-Lys(Y)-Pro-Arg-OH	Gly	18.40	55
9c		H	Arg-Pro-Lys(Y)-Thr-OH	H	18.59	48
9d		H	Arg-Pro-Lys(Y)-Thr-OH	Gly	19.02	54
9e		H	Arg-Pro-Lys(Y)-Thr-OH	Ile	19.00	58
9f		NO_2	Thr-Lys(Y)-Pro-Arg-OH	H	19.19	46
9g		NO_2	Thr-Lys(Y)-Pro-Arg-OH	Ile	19.32	56
9h		NO_2	Arg-Pro-Lys(Y)-Thr-OH	H	19.39	51
9i		NO_2	Arg-Pro-Lys(Y)-Thr-OH	Ile	19.44	61

adenocarcinoma A549 cells and myeloid leukemia HL-60 cells. As shown in Table 3, some conjugates were not cytotoxic at concentrations up to 100 μM (**4f**, **4g**, **4i**, **9b**, **9d**, **9e**). Other studied compounds (both precursors and tuftsin conjugates) showed only very modest cytotoxicity at high micromolar concentrations (Table 3). However, for compound **7d** conjugation with tuftsin analogue but not tuftsin itself led to about 2-fold increased cytotoxicity toward both tumor cell types compared to the respective precursor **5b**. In contrast, compound **7a** was about 6-fold more cytotoxic toward A549 cells than its nonpeptidic precursor **5a** and showed comparable cytotoxicity toward HL-60 cells. In some situations, tuftsin conjugates possess similar or decreased cytotoxic activity compared to their precursors (compare cytotoxic activities of conjugates **7e**, **7g**, **7h** and compound **5b** or **4f**, **4g**, **4i** and precursor **4** in Table 3).

Table 3. In Vitro Cytotoxicity ($IC_{50} \pm SD$ (μM)) of Selected Conjugates against Human Myeloid Leukemia HL-60 and Lung Adenocarcinoma A549 Cells[a]

				cytotoxicity (IC_{50} in μM) human cancer cell lines	
compd	*n*	X	R	HL-60	A549
9		H	OH	>100	>100
9b		H	Thr-Lys(Gly)-Pro-Arg-OH	>100	>100
9d		H	Arg-Pro-Lys(Gly)-Thr-OH	>100	>100
9e		H	Arg-Pro-Lys(Ile)-Thr-OH	>100	>100
8a		NO_2	OH	>100	>100
9g		NO_2	Thr-Lys(Ile)-Pro-Arg-OH	>100	>100
9i		NO_2	Arg-Pro-Lys(Ile)-Thr-OH	>100	38.9 ± 3.6
5b	5	CH_3	OH	17.5 ± 1.1	18.7 ± 1.4
7b	5	CH_3	Thr-Lys(Gly)-Pro-Arg-OH	55.5 ± 6.2	76.5 ± 5.9
7d	5	CH_3	Thr-Lys(Ile)-Pro-Arg-OH	11.3 ± 0.7	8.7 ± 1.8
7e	5	CH_3	Thr-Lys-Pro-Arg-OH	13.2 ± 0.9	15.0 ± 2.3
7g	5	CH_3	Arg-Pro-Lys(Ile)-Thr-OH	23.2 ± 2.5	47.3 ± 5.4
7h	5	CH_3	Arg-Pro-Lys-Thr-OH	22.1 ± 1.8	25.6 ± 6.1
5a	3	CH_3	OH	15.5 ± 0.8	17.6 ± 2.2
7a	3	CH_3	Thr-Lys(Gly)-Pro-Arg-OH	17.2 ± 2.7	3.0 ± 0.6
5c	2	H	OH	18.9 ± 1.4	6.0 ± 1.7
7f	2	H	Arg-Pro-Lys(Gly)-Thr-OH	71.5 ± 3.6	33.9 ± 4.1
4			OH	32.4 ± 3.1	14.2 ± 1.6
1				15.8 ± 1.3	15.8 ± 1.9
4f			Arg-Pro-Lys-Thr-OH	>100	>100
4g			Arg-Pro-Lys(Ala)-Thr-OH	>100	>100
4i			Arg-Pro-Lys(Val)-Thr-OH	nd	41.5 ± 4.2

[a] nd: not determined.

To clarify why precursors **8a** and **9** were not cytotoxic to tumor cells, we analyzed the protonation status of these compounds at physiological pH using the SPARC online server. This analysis allows one to calculate lipophilicity parameter log *P* and fractions of nonprotonated/protonated compounds at different pH conditions.[27] At pH 7, both compounds **8a** and **9** were completely deprotonated and carried negative charge (data not shown) that most likely precluded their transport through the plasma membrane by passive diffusion. Other precursor compounds either were not protonated at physiological pH (compounds **1** and **4**) or were present mostly (about 80% molecules) as zwitterions (compounds **5a**−**c**).

Analysis of the structure−activity relationship for studied compounds led us to conclude that the most important parameter that influences the cytotoxic activity of tuftsin conjugates with different chromophores is the type of a peptide and the way it is conjugated to respective precursors (type of the chemical linker). Comparison of different compounds with −COR linkers, which differ only by attached peptides, revealed that the highest cytotoxic activity toward A549 cells was observed for compound **7d**, which contains tuftsin with branched isoleucin molecule. In contrast, its close analogue compound **7e** with attached tuftsin had about 2-fold lower cytotoxicity (see Table 3). Interestingly, conjugation of compound **5b** with the same type of linear peptide but different branching amino acid (tuftsin with branched glycine residue) resulted in compound **7b** that showed about 3- to 4-fold lower cytoxicity toward both tumor cell types compared to compound **5b**.

Within the group of conjugates with tuftsin and its analogues combined with different procursors by a −CONHR linker, two compounds **7a** and **7b**, with the same peptide (tuftsin with branched glycine residue) but different precursors (either **5a** or **5b**), had cytotoxic activities toward A549 cells that differed by about 25-fold, where conjugate **7a** with precursor **5a** was the most cytotoxic. It is worth noting that compounds **7a** and **7b** differed also in the length of the linker between the chromophore moiety and tuftsin analogue in terms of the number of methylene residues (three and two, respectively). Since the conjugate with a shorter linker (compound **7b**) was much less cytotoxic than its structural analogue compound **7a**, it strongly suggests that the attachment of precursors through a long linker increases biological activity of tuftsin conjugates. Equally important, compound **7a** was also more cytotoxic (by about 6-fold) toward A549 cells than its precursor **5a**. Together, our data show that covalent conjugation of branched tuftsin and one type of studied chromophores, namely, nitroacridine moiety, resulted in compounds with greatly increased cytotoxic activity compared to its chromophore precursor. Most interestingly, we also show that this increased cytotoxic effect was specific for lung adenocarcinoma A549 cells and not observed in HL-60 myeloid leukemia cells.

It is not clear why several tuftsin conjugates showed increased cytotoxicity toward A549 cells compared to HL-60 cells. One of the possibilities can be that A549 cells possess high levels of tuftsin receptors. Unfortunately, the expression levels of tuftsin receptors in tumor cells have not been systematically explored. On the other hand, tuftsin receptor has been purified from myeloid leukemia HL-60 cells and it is likely that it is expressed in other tumor cells; however, there are no tools available (e.g., antibodies directed to tuftsin receptor) to experimentally verify its expression in tumor cells.[28] Second, the native amino acid sequence of tuftsin is present in the Fc portion of IgG protein.[29] Recent studies showed that multivalent forms of a 20-amino acid

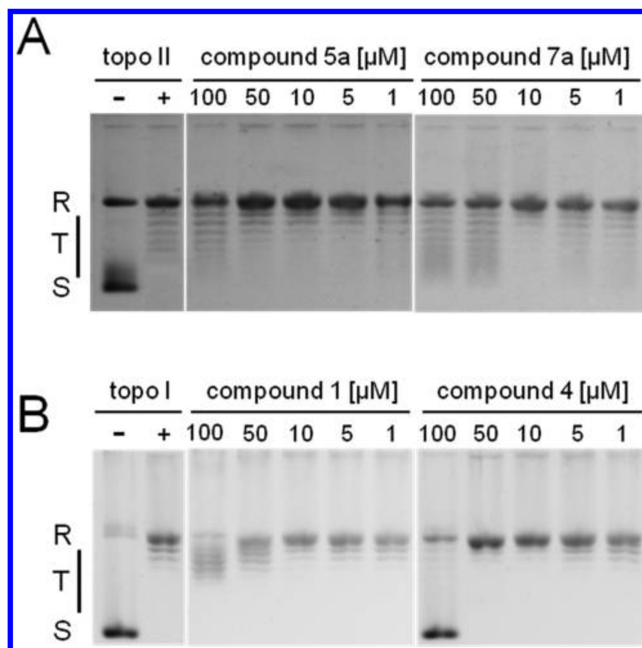

Figure 1. Inhibition of the catalytic acitvity of type I and type II DNA topoisomerases by studied compounds: (A) effect of acridine **5a** and its tuftsin conjugate compound **7a** on the topoisomerase II mediated relaxation of DNA; (B) effect of two anthraquinone-based compounds, **1** and **4**, on DNA relaxation mediated by type I topoisomerase. S and R indicate supercoiled and relaxed DNA, respectively, and T is DNA topoisomers.

EC-1 peptide, containing Fc fragment of the human IgG, avidly bind a member of the epidermal growth factor (EGF) receptor family, ErbB2, in breast carcinoma SK-BR-3 cells,[30] which leads to its enhanced internalization and endosomal accumulation. Since EGF receptors are frequently abnormaly expressed in several types of human tumors, including lung tumors,[31] one may speculate that increased sensitivity of lung adenocarcinoma A549 cells to tuftsin conjugate **7a** may be related to higher expression of EGF receptors in these cells.

Inhibition of the Catalytic Activity of Human Types I and II DNA Topoisomerases. Previous studies showed that peptide−anthraquinone analogues inhibit the catalytic activity of DNA topoisomerase I and stabilize the so-called "cleavable complexes" between DNA and the enzyme.[21] Therefore, we determined if drug−tuftsin conjugates exert a similar effect on the activity of purified types I and II DNA topoisomerases. Our results show that none of the studied compounds, both precursor derivatives and tuftsin conjugates, significantly influenced the DNA relaxation mediated by both type I and type II DNA topoisomerases (Figure 1A, data not shown). This can be related to the presence of a negative charge at physiological pH in compound **8a** and both negative and positive charges in compounds **5a**−**c**. This is supported by the fact that two precursors, compounds **1** and **4**, which are not protonated/deprotonated at physiological pH, partially inhibited DNA topoisomerase I activity at high concentrations (Figure 1B). Therefore, it is possible that the presence of electrostatic charge at molecules of precursors or conjugates prevents an effective binding of these compounds to DNA and their interaction with DNA topoisomerases.

Effect of Studied Compounds on Cell Cycle Progression and Induction of Cell Death of Tumor Cells. We also analyzed

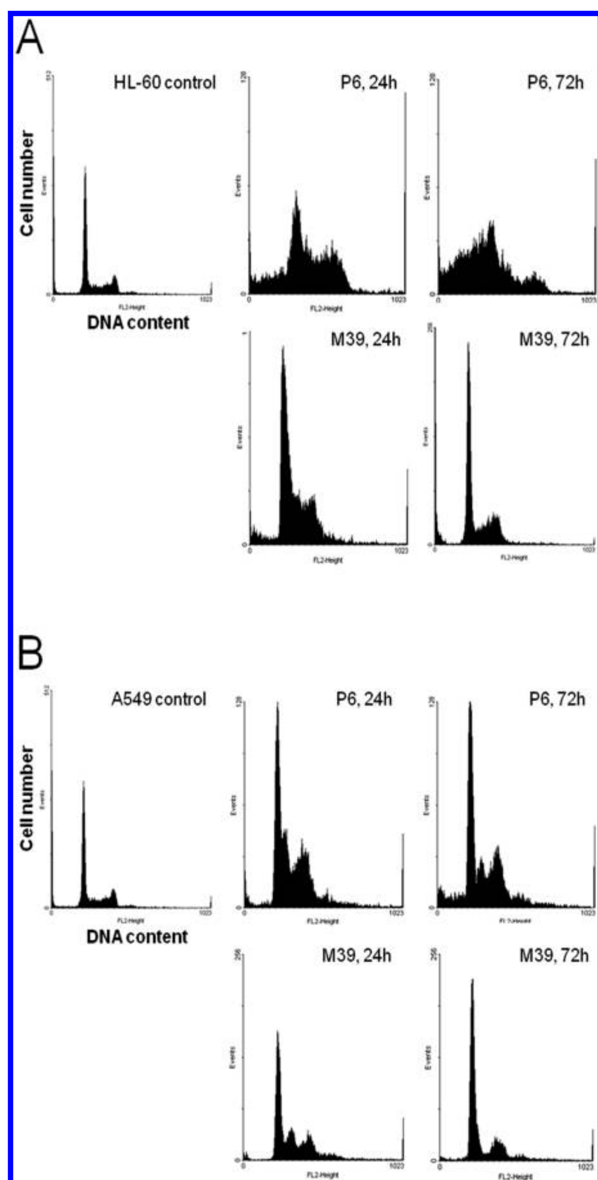

Figure 2. Effect of acridine **5a** and its tuftsin conjugate compound **7a** on the cell cycle progression of HL-60 (myeloid leukemia) and A549 (lung adenocarcinoma) tumor cells exposed to the isotoxic concentrations corresponding to their IC_{90} doses for different time periods.

the effect of the studied compounds on cell cycle progression of both types of tumor cells at equitoxic concentrations corresponding to the IC_{90} concentrations. Biologically active conjugates arrested cells in the early S phase and in G2 and M of the cell cycle. Exposure of both A549 and HL-60 cells to studied precursors led to emptying of the S phase. Exemplary results for **5a** precursor and the most cell type specific and cytotoxic **7a** conjugate are shown in Figure 2, panels A and B.

We then wanted to establish the type of cell death induced by studied compounds. To this end, we analyzed the nuclear morphology of tumor cells exposed to precursor **5a** and conjugate **7a** and revealed that cell death was induced differently depending on the type of tumor cells. In HL-60 cells, precursor **5a** induced rapid apoptosis, whereas no apoptotic figures were observed in cells incubated with conjugate **7a** (Figure 3). Interestingly, no apparent changes in nuclear morphology were

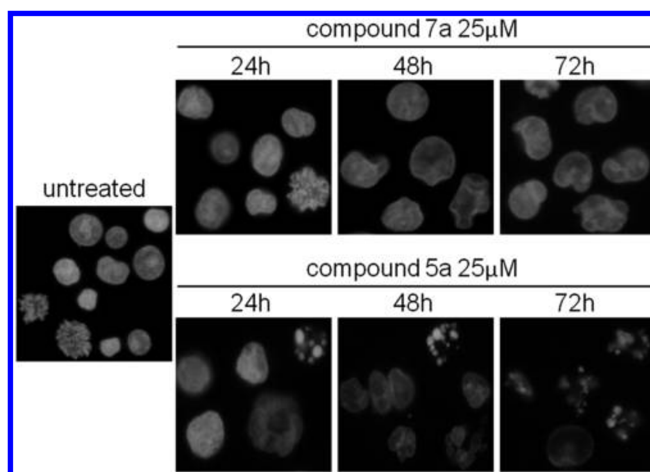

Figure 3. Cell-death-related changes in nuclear morphology of HL-60 cells exposed to acridine **5a** and its tuftsin conjugate compound **7a** at the isotoxic concentrations corresponding to their IC_{90} doses for different time periods. Following treatment, cells were stained with Hoechst 33342 dye and analyzed by fluorescent microscopy as described in Experimental Section. Original magnification is ×600.

observed in A549 cells exposed to both precursor **5a** and conjugate **7b** (not shown), suggesting that in these cells studied compounds do not induce morphological changes typical of apoptosis.

CONCLUSION

In this paper we describe a new series of peptidic conjugates that contain tuftsin or its linear or branched analogues combined with anthracenediones and acridone/acridines. We showed that several conjugates possess increased and specific cytotoxic activity toward human tumor cells compared to its precursor. These results confirmed our initial idea that by combining a peptidic component based on tuftsin and its analogues, which serve as a drug delivery system, with small molecules, one may increase the cytotoxicity and specificity of compounds that otherwise are only marginally biologically active. Further studies will show whether drug−tuftsin conjugates possess similar tumor selectivity and low toxicity in in vivo animal models.

EXPERIMENTAL SECTION

Chemistry. The composition and purity of all compounds were confirmed by MS, ^1H NMR, and UV−vis spectra, elemental analysis, and analytical RP-HPLC. All tuftsin conjugates were obtained in good yield (31−45% for anthraquinone derivatives, 38−52% for acridine derivatives, and 43−61% for acridone derivatives) and ≥95% purity as revealed using RP-HPLC analysis. In RP-HPLC, the tuftsin conjugates were examined with a linear gradient of 0−90% of solvent B over 30 min (type A) and a linear gradient of 0−100% of solvent B over 60 min (types B and C).

Mass spectra of synthesized conjugates were recorded using matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry (MALDI-TOF MS, Biflex III Bruker). ^1H NMR spectra were measured in dimethylsulfoxide (DMSO) with a Varian 500 NMR spectrometer. UV−vis spectra were recorded on a Gallab 772 spectrophotometer. Elemental analysis was carried out with a Carlo Erba EA 1108 analyzer. RP-HPLC procedures were performed using a Beckman Gold System chromatograph. The analytical column employed was a Kromasil C_8 column (4.6 mm × 250 mm, 5 μm, 100 Å). Chromatographic separation

was performed on a Kromasil C_8 semipreparative column (10 mm × 250 mm, 5 μm, 100 Å). Cartridges (6 mL, 15 mL) packed with C_{18} resin were used for SPE. HPLC or SPE solvent system was as follows: solvent A, 0.1% TFA/H_2O; solvent B, 0.1% TFA/CH_3CN/H_2O (80:20, v/v). TLC solvent system was as follows: (a) *n*-Bu-AcOH-H_2O-AcOEt (1:1:1:1, v/v/v/v), (b) AcOEt-EtOH-H_2O-NH_3 (5:3:2:0.5, v/v/v/v). Detection was carried out using UV and ninhydrin. All compounds were monitored on Merck F_{254} silica gel precoated plates. All chemicals and solvents were of reagent grade and were used without further purification. The acridine derivatives (**5a**−**c**) were prepared as described elsewhere.[32−35] The 4-carboxyacridones (**8a**, **8b**) were obtained according to a well-known procedure.[35−37] The leuco-1,4-dihydroxyanthraquionone (**1**) was given as described.[23]

General Procedure for Synthesis of Peptide-Resin. The resin (1 g, Wang/TentaGel S PHB) was wetted with dimethylene chloride (DCM) (10 mL) for 2 h and filtrated. Then Fmoc-amino acid-OH (2.50 mmol) and HOBt (0.80 mmol) were dissolved in the mixture of DCM and DMF (5 mL, 9:1, v/v) and added to resin. 4-Dimethylaminopyridine (0.08 mmol) was dissolved in DMF and added to resin as well. Next DIC (123.90 μL) was added to resin and the suspension was shaken for 5 h. At the end of this time, the resin was filtrated and shaken with a mixture of acetic anhydrate and pyridine (1:1, v/v) for 1 h. Then the resin was filtered, washed three times with DMF, DCM, MeOH, and dried under reduced pressure. The prepared resin was wetted with DCM and treated with 20% piperidine in DMF (10 mL) for 5 and 15 min. Then the resin was filtered and washed with DCM and DMF. Each coupling step was performed with Fmoc-AA-OH (0.65 mmol) diluted in a mixture of DMF, *N*-methylpirolidone (NMP), and DCM (10 mL, 1:1:1, v/v/v) in the presence of 1% Triton, using DIC (0.65 mmol) and HOBt (0.65 mmol) for 90 min. Next, the resin was filtered and washed with DMF and DCM. The cycle of the two-step procedure (deprotection and coupling) was repeated until the expected peptide sequence was obtained. Then the prepared tuftsin−resin was treated with 5% TFA in DCM and was washed with 10% DIPEA in DCM and DCM. Free ε-amino group of lysine was attached to Boc-AA-OH (0.65 mmol) using TBTU (0.65 mmol), HOBt (0.65 mmol) in the presence of DIPEA (1.30 mmol), and the sample was shaken for 45 min. The progress of couplings was monitored by chloranil test. The final deprotection was achieved by treatment of 20% piperidine in DMF, and then the resin was washed with DMF, DCM, and diethyl ether. The prepared resin was dried under reduced pressure.

General Procedure for Synthesis of Anthraquinone Tuftsin Analogues (4a−k). To a solution of 187 mg (0.78 mmol) of compound **1** in DMF (25 mL), 1 g (0.26 mmol) of peptide−resin was added and heated to 120 °C into the oil bath for 24 h with stirring under nitrogen. Then acetone (208 mL) was added to the mixture and peptidyl-anthraquinone-resin was oxidized for 6−8 h. Later, the peptidyl-anthraquinone-resin was filtrated and washed with DMF, acetone, DCM, and diethyl ether.[20] The prepared resin was dried under reduced pressure. Next, the peptidyl-anthraquinone-resin was treated with a cocktail of TFA, H_2O, and TIS (95:2.5:2.5, v/v/v) for 2.5 h. The cleaved compound was precipitated with diethyl ether and lyophilized from water. The crude product was purified by SPE or HPLC to obtain highly pure (≥95%) dark blue powder.

General Procedure for Synthesis of Tuftsin−Acridine/Tuftsin−Acridone Conjugates. To a solution of 1-nitroacridine (**5**) (0.7 mmol) or 4-carboxyacridone (**8**) (0.7 mmol) derivatives, TBTU (1.4 mmol), and HOBt (1.4 mmol) in anhydrous DMF, the resin (0.35 mmol/g) was added. After 3 min, DIPEA (2.8 mmol) was added into the reaction medium. The mixture was shaken for 48 h at room temperature. Then the resin was filtrated, washed with DMF, DCM, diethyl ether, and dried under reduced pressure. The peptidyl-conjugate-resin was treated with a mixture of TFA, H_2O, and TIS (95:2.5:2.5, v/v/v) for 3.5 h. The cleaved compound was precipitated with diethyl ether and lyophilized. The crude product was purified by SPE or HPLC to obtain highly pure (≥95%) yellow powder.

***N*-[(4-Methyl-1-nitro-9-acridinyl)-γ-aminopropanoyl]-Thr-Lys(Gly)-Pro-Arg-OH (7a).** To a solution of **5a** (0.7 mmol) derivative, TBTU (1.4 mmol), and HOBt (1.4 mmol) in anhydrous DMF, Thr-Lys(Gly)-Pro-Arg-resin (0.35 mmol/g) was added. After 3 min, DIPEA (2.8 mmol) was added into the reaction medium. The mixture was shaken for 48 h at room temperature. Then the resin was filtrated, washed with DMF, DCM, diethyl ether, and dried under reduced pressure. The peptidyl-conjugate-resin was treated with a mixture of TFA, H_2O, and TIS (95:2.5:2.5, v/v/v) for 3.5 h. The cleaved compound was precipitated with diethyl ether and lyophilized. The crude product was purified by SPE or HPLC to obtain highly pure (≥95%) yellow powder: yield 38%; MS $[M + H]^+$ 879.2; R_f = 0.32 (solvent b); UV−vis (H_2O) λ (log ε) 422 (3.63), 430 (3.67), 460 (2.55). Anal. ($C_{43}H_{59}N_{12}O_{12}F_3 \cdot H_2O$) C, H, N.

***N*-[(4-Methyl-1-nitro-9-acridinyl)-β-alanyl]-Thr-Lys(Gly)-Pro-Arg-OH (7b).** To a solution of **5b** (0.7 mmol) derivative, TBTU (1.4 mmol), and HOBt (1.4 mmol) in anhydrous DMF, Thr-Lys(Gly)-Pro-Arg-resin (0.35 mmol/g) was added. After 3 min, DIPEA (2.8 mmol) was added into the reaction medium. The mixture was shaken for 48 h at room temperature. Then the resin was filtrated, washed with DMF, DCM, diethyl ether, and dried under reduced pressure. The peptidyl-conjugate-resin was treated with a mixture of TFA, H_2O, and TIS (95:2.5:2.5, v/v/v) for 3.5 h. The cleaved compound was precipitated with diethyl ether and lyophilized. The crude product was purified by SPE or HPLC to obtain highly pure (≥95%) yellow powder: yield 50%; MS $[M + H]^+$ 865.6; R_f = 0.38 (solvent b); UV−vis (H^2O) λ (log ε) 420 (3.50), 440 (3.52), 460 (3.35); ^{1}H NMR δ 1.12 (d, *J* = 6.0 Hz, 3H, T-CH_3), 1.32 (m, 2H, K-γCH_2), 1.54 (m, 2H, K-δCH_2), 1.55 (m, 2H, R-γCH_2), 1.56 (m, 1H, K-βCH), 1.60 (m, 1H, R-βCH), 1.66 (m, 1H, K-βCH), 1.74 (m, 2H, R-γCH_2), 1.74 (m, 1H, R-βCH), 1.83 (m, 1H, P-βCH), 1.86 (m, 2H, P-γCH_2), 2.07 (m, 1H, P-βCH), 2.42 (t, 2H, *J* = 6.4 Hz, NHCH_2CH_2), 2.45 (s, 3H, C4-CH_3), 2.74 (m, 2H, K-εCH_2), 3.01 (t, 2H, *J* = 6.5 Hz, CH_2CH_2CO), 3.10 (m, 2H, R-γCH_2), 3.53 (m, 1H, P-δCH), 3.56 (m, 2H, G-αCH_2), 3.66 (m, 1H, P-δCH), 4.02 (m, 1H, T-βCH), 4.12 (m, 1H, R-αCH), 4.28 (t, *J* = 6.2 Hz, 1H, T-αCH), 4.34 (m, 1H, P-αCH), 4.53 (m, 1H, K-αCH), 5.07 (bd, 1H, T-OH), 7.31 (t, *J* = 7.5 Hz, 1H, C7-H), 7.51 (d, *J* = 8.1 Hz, 1H, C2-H), 7.52 (m, 2H, G-αNH_2), 7.54 (t, *J* = 5.6 Hz, 1H, R-δNH), 7.58 (d, *J* = 8.7 Hz, 1H, C3-H), 7.70 (t, 1H, *J* = 7.7 Hz, C6-H), 7.81 (d, J = 8.0 Hz, 1H, C5-H), 7.82 (t, J = 5.3 Hz, 1H, K-εNH), 7.95 (d, *J* = 8.4 Hz, 1H, C8-H), 8.18 (d, *J* = 7.6 Hz, 1H, R-αNH), 8.28 (m, 1H, K-αNH), 9.15 (brs, 1H, CONH), 10.26 (d, *J* = 7.9 Hz, 1H, T-αNH).

***N*-[(4-Methyl-1-nitro-9-acridinyl)-ε-aminopentanyl]-Thr-Lys(Ile)-Pro-Arg-OH (7d).** To a solution of **5c** (0.7 mmol) derivative, TBTU (1.4 mmol), and HOBt (1.4 mmol) in anhydrous DMF, Thr-Lys(Ile)-Pro-Arg-resin (0.35 mmol/g) was added. After 3 min, DIPEA (2.8 mmol) was added into the reaction medium. The mixture was shaken for 48 h at room temperature. Then the resin was filtered, washed with DMF, DCM, diethyl ether, and dried under reduced pressure. The peptidyl-conjugate-resin was treated with a mixture of TFA, H_2O, and TIS (95:2.5:2.5, v/v/v) for 3.5 h. The cleaved compound was precipitated with diethyl ether and lyophilized. The crude product was purified by SPE or HPLC to obtain highly pure (≥95%) yellow powder: yield 48%; MS $[M + H]^+$ 963.1; R_f = 0.37 (solvent b); UV−vis (H^2O) λ (log ε) 400 (3.58), 420 (3.79), 460 (3.63); ^{1}H NMR δ 0.96 and 1.04 (2d, *J* = 8.2 Hz, 6H, I-CH_3), 1.13 (m, 10H, $NH(CH_2)_5$), 1.25 and 1.28 (m, 2H, I-γCH_2), 1.21 (d, *J* = 6.1 Hz, 3H, T-CH_3), 1.35 (m, 2H, K-γCH_2), 1.52 (m, 2H, K-δCH_2), 1.53 (m, 1H, R-γCH_2), 1.54 (m, 1H, K-βCH), 1.60 (m, 1H, R-βCH), 1.66 (m, 1H, K-βCH), 1.74 (m, 1H, R-γCH_2), 1.74 (m, 1H, R-βCH), 1.83 (m, 1H, P-βCH), 1.88 (m, 2H, P-γCH_2), 2.07 (m, 1H, P-βCH), 2.10 (m, 1H, I-βCH), 2.15 (m, bs, 2H I-αNH_2), 2.35 (s, 3H, C4-CH_3), 2.72 (m, 2H, K-εCH_2), 3.10 (m, 2H, R-γCH_2),

3.53 (m, 1H, P-δCH), 3.61 (m, 1H, I-αCH), 3.66 (m, 1H, P-δCH), 4.02 (m, 1H, T-βCH), 4.12 (m, 1H, R-αCH), 4.31 (t, J = 6.4 Hz, 1H, T-αCH), 4.36 (m, 1H, P-αCH), 4.53 (m, 1H, K-αCH), 5.09 (bd, 1H, T-OH), 7.34 (t, J = 7.49 Hz, 1H, C7-H), 7.53 (d, J = 8.7 Hz, 1H, C3-H), 7.56 (t, J = 5.4 Hz, 1H, R-δNH), 7.58 (d, J = 7.4 Hz, 1H, C2-H), 7.59 (t, J = 7.0 Hz, 1H, C6-H), 7.80 (2d, J = 8.3 Hz, J = 8.1 Hz, 2H, C5-H, C8-H), 7.88 (t, J = 5.3 Hz, 1H, K-εNH), 8.18 (d, J = 7.6 Hz, 1H, R-αNH), 8.28 (m, 1H, K-αNH), 9.12 (bs, 1H, CONH), 10.17 (d, J = 7.9 Hz, 1H, T-αNH).

Drugs and Chemicals. All compounds were dissolved in DMSO (10 mM stock solutions) and were kept at −20 °C until use. All other reagents were of analytical grade.

DNA Substrates and Enzymes. Supercoiled plasmid pBR322 DNA (>95% form I) and human type I topoisomerase were purchased from Invitrogen. Human type IIα topoisomerase was isolated from *Saccharomyces cerevisiae* overexpressing a multicopy plasmid and kindly provided by Prof. Anni H. Andersen (University of Aarhus, Denmark).

Calculations of log *P*. The lipophilicity parameter log P value was calculated for studied precursors using the SPARC (predictive modeling system) online server at the U.S. Environmental Protection Agency (Research Triangle Park, NC).[27] Calculated log P values were obtained for partition of drugs between water and octanol at 25 °C/760 Torr.

Inhibition of DNA Relaxation Mediated by Topoisomerases. The reaction mixture contained 20 mM Tris-HCl, pH 7.5, 7.5 mM $MgCl_2$, 0.5 mM DTT, 150 mM KCl, 1 mM ATP, and 200 ng of pBR322 DNA. The reaction was initiated by the addition of DNA topoisomerase II and studied compounds and allowed to proceed at 37 °C for 30 min. Reactions were terminated by addition of loading buffer (0.1% SDS, 0.05% bromophenol blue, 2.5 mM EDTA, 10% sucrose, final concentrations). The samples were separated in 1.2% agarose gels at 0.5 V/cm for 18 h in TBE buffer (90 mM Tris-borate, 1 mM EDTA, pH 8). Gels were stained with 0.5 μg/mL ethidium bromide to visualize DNA and were photographed under UV illumination as described previously.[38] The conditions for DNA relaxation assay for type I topoisomerase were the same except different reaction buffer was used (10 mM Tris-HCl, pH 7.5, 7.5 mM $MgCl_2$, 60 mM KCl).

Cell Lines. HL-60 leukemia and A549 lung carcinoma cells were purchased from American Type Culture Collection (Rockville, MD). The cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and antibiotics (100 units/mL penicillin and 100 μg/mL streptomycin) at 37 °C in 5% CO_2/air atmosphere. All cell lines were screened routinely for Mycoplasma by the PCR method with Mycoplasma Plus PCR primer set (Stratagene, La Jolla, CA).

Cytotoxicity Assays. The cytotoxicity was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, exponentially growing cells were continuously exposed to different drug concentrations and the cellular viability was determined after three to four generation times. Cells were exposed to the MTT tetrazolium salt for 4 h at 37 °C, and the formation of formazan was measured by a microplate reader. The concentrations required to inhibit cell growth by 50% compared to untreated controls were determined from the curves plotting survival as a function of dose by use of the Slide Write program. All values are averages of at least two independent experiments, each done in duplicate.

Flow Cytometry Analysis of Cell Cycle Distribution. Distribution in different phases of the cell cycle was analyzed after treatment of tumor cells with studied precursors and respective conjugates. Briefly, following drug treatment, cells were washed in ice-cold phosphate buffered saline (PBS) and fixed in 80% ethanol at −24 °C. Cells were stained in PBS containing 20 μg/mL propidium iodide and 100 μg/mL ribonuclease A for 30 min at room temperature. Samples were analyzed by a FACScan flow cytometer (Becton Dickinson) equipped with a 488 nm laser, and the distribution of cells in the cell cycle was calculated using MultiCycle software (Phoenix Flow Systems, San Diego, CA).

Nuclear Morphology of Tumor Cells. Cells were attached to cover slides in 35 mm Petri dishes and treated with studied compounds for different time periods. Following drug treatment, cells were stained with 1 μg/mL Hoechst 33342 dye for 15 min and analyzed by Olympus BX-60 fluorescent microscope equipped with respective optical filters. Images were recorded using DP-50 digital camera and image acquisition software Viewfinder Light, version 1.0 (Pixora Corporation, U.S.).

■ ASSOCIATED CONTENT

Ⓢ Supporting Information. Analytical and spectroscopic data for compounds **4a**−**k**, **7a**−**h**, and **9a**−**i**. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS USED

AA, amino acid; Boc, *tert*-butoxycarbonyl; DCM, methylene chloride; DIC, *N*,*N*′-diisopropylocarbodiimide; DIPEA, *N*,*N*-diisopropylethylamine; DMF, *N*,*N*-dimethylformamide; DMSO, dimethylsulfoxide; EGF, epidermal growth factor; Fmoc, fluorenylmethoxycarbonyl; HOBt, *N*-hydroxybenzotriazole; IgG, immunoglobulin G; Mtt, 4-methyltrityl; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NMP, *N*-methylpirolidone; PBS, phosphate buffered saline; Pfb, 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl; SPE, solid-phase extraction; SPPS, solid-phase peptide synthesis; TBTU, *O*-benzotriazol-1-yl-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate; ^{t}Bu, *tert*-butyl; TFA, trifluoroacetic acid; TIS, triisopropylsilane

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