New Daunomycin–Oligoarginine Conjugates: Synthesis, Characterization, and Effect on Human Leukemia and Human Hepatoma Cells

Zsanett Miklán,[1] Erika Orbán,[1] Gabriella Csík,[2] Gitta Schlosser,[3,4] Anna Magyar,[1] Ferenc Hudecz[1,4]

[1] *Research Group of Peptide Chemistry, Hungarian Academy of Sciences, Eötvös Loránd University, Budapest, Hungary*

[2] *Department of Biophysics and Radiobiology, Semmelweis Medical University, Budapest, Hungary*

[3] *Philips Research Europe Laboratories, Aachen, Germany*

[4] *Department of Organic Chemistry, Eötvös Loránd University, Budapest, Hungary*

Received 16 March 2009; revised 22 May 2009; accepted 26 May 2009
Published online 11 June 2009 in Wiley InterScience (www.interscience.wiley.com). DOI 10.1002/bip.21264

ABSTRACT:

In this article, the synthesis, a novel chromatographic procedure and characteristics of a new class of daunomycin (Dau)–oligoarginine conjugates are described. In these compounds oligoarginine with 6 or 8 residues (Arg_n, $n = 6, 8$) is attached to Dau by different covalent bond: squaric amide (Dau-□-Arg_n), oxime (Dau=N—O—CH_2—CO—Arg_n), or hydrazone (H—Glu(Arg_n)—NH—N=Dau). Conjugates were characterized by RP-HPLC and mass spectrometry. We report also on our findings concerning chemical and biological properties of Dau-conjugates as a function of covalent linkage, site of conjugation and length of the oligoarginine moiety. Stability, fluorescent properties as well as cytostatic effect and cellular uptake of these compounds were studied. Dau-conjugates with squaric amide or oxime linkage were stable, but continuous release of free Dau was observed from the hydrazone conjugate in solution. We found that some spectral characteristics (e.g., the amplitude of the emission spectrum) of conjugates could be sensitive for the site of coupling (amino vs. oxo function). Cytostasis and cellular uptake of conjugates were investigated both on human leukemia (HL-60) and human hepatoma (HepG2) cell lines by MTT assay and flow cytometry We found that cytostatic effect and uptake properties of Dau-conjugates were dependent on the acid stability of the linkage (hydrazone vs. oxime/amide) applied and more markedly on the cell line studied. © *2009 Wiley Periodicals, Inc. Biopolymers (Pept Sci) 92: 489–501, 2009.*

Keywords: *daunomycin; oligoarginine conjugates; synthesis of daunomycin conjugates; in vitro cytostatic effect; fluorescence properties; effect of covalent linkage type*

Correspondence to: Ferenc Hudecz; e-mail: fhudecz@elte.hu
Zsanett Miklán and Erika Orbán contributed equally to this work.
Contract grant sponsor: Hungarian Research Fund (OTKA)
Contract grant number: K68285
Contract grant sponsor: Hungarian Ministry of Health
Contract grant number: ETT 459/2006
Contract grant sponsor: National Office for Research and Technology, Hungary
Contract grant numbers: 3.2.1-2004-04-0005/3.0, 3.2.1-2004-04-0352/3.0
Contract grant sponsors: Magyary Zoltán Higher Educational Public Foundation (Hungary) and Marie Curie Actions of the European Commission (Project Nr. 14457, Diagnostic Peptide Pattern for Early Stage Disease Detection)

This article was originally published online as an accepted preprint. The "Published Online" date corresponds to the preprint version. You can request a copy of the preprint by emailing the Biopolymers editorial office at biopolymers@wiley.com

INTRODUCTION

Daunomycin (Dau) is an anthracycline type antibiotic and also widely used for the treatment of cancer. Clinical application of Dau is limited by side-effects such as immunsuppression, nephrotoxicity, and cardiotoxicity. The intrinsic or acquired resistance of tumor cells to Dau also reduces the response to the treatment. Conjugation with different type of carrier (e.g., oligo- and polypeptides,[1] proteins,[2] polysaccharides,[3] polymers,[4,5] dextran[6]) could decrease the side-effects and improve the selectivity of the drug attached by utilization of different cellular uptake mechanism(s).

Dau has four functional groups suitable for covalent conjugation (Figure 1). According to the literature, the most frequently applied site is the primary amino group of the carbohydrate moiety even though the free amino group is a critical determinant of DNA binding ability.[7] If this amino group is used for conjugation, the drug has to be released from the conjugate inside the cell to liberate the amino group. The first conjugations of anthracyclines on this site were performed in the 1970s by using glutaraldehyde and periodate oxidation or *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester linkage as reported by Baurain et al.[2] The maleimide linkers are frequently applied not only with the amino group.[8–10] Amide bond formation can also be utilized assuming that this bond could be hydrolyzed by lysosomal[2,3,11] or organ-specific enzymes.[12] In other cases, acid-labile linkers were introduced between the anthracycline and carrier so the drug could be released under acidic circumstances (e.g., the inside of lysosomes). *Cis*-aconityl,[13,14] succinyl,[15,16] various diacidic[17] spacers, and also dimethylmaleic anhydride[5] were applied for this purpose. Conjugates of anthracyclines were synthesized also with formaldehyde[18] or estrogen.[19] Furthermore, squaric amide[20] and carbamate[21] derivatives were also prepared as promising prodrugs.

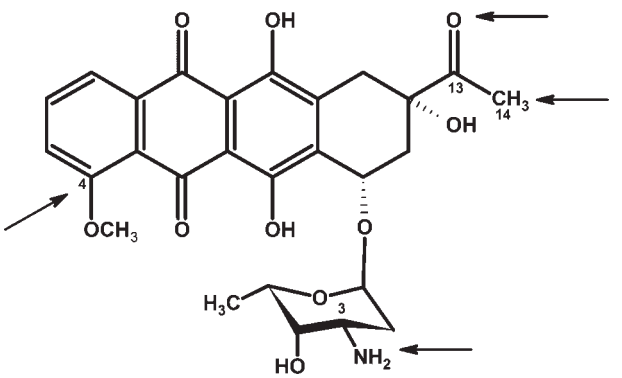


FIGURE 1 Structure of daunomycin indicating four functions suitable for covalent conjugation.

Modification of anthracyclines is also possible on the 13-oxo group using different hydrazide[8,10,22,23] and *O*-alkyl-hydroxylamine[22,24] derivatives.

The replacement of the methyl ether group on C-4 provides another option for conjugation. This strategy was used recently for coupling of Dau to triplex forming oligonucleotide(s).[25] By the synthesis of the 14-bromo-derivative of Dau, conjugates with ester or tioether bond could also be generated.[26,27] Peptide conjugates of Dau were synthesized for various purposes: to reduce its side-effects;[22] to circumvent multidrug resistance;[27,28] to increase selectivity or target the molecule to specific part of the body.[8,12,15,29]

The nature of chemical bond between Dau and the peptide component could markedly influence the chemical and biological properties of the conjugate and thus the efficacy of the drug. Interestingly, no related systematic studies were published with Dau oligopeptide conjugates.

Oligoarginines (Arg_n) belong to the group of cell-penetrating peptides (CPP). They were developed after the discovery that polycationic, Arg-rich peptides from different origin (e.g., HIV-Tat, Antennapedia) are able to translocate the cell membrane.[30,31] It was found that oligoarginines can be taken up more effectively than other cationic oligopeptides with equal length, e.g., oligomers of 3-9 Lys, Orn, His.[32] It was also proved that an optimum number of Arg-residue is required for effective internalization.[33] The chirality of Arg-oligomers has limited influence on translocation, but the guanidine head group and its distance from the peptide backbone could influence the intracellular translocation.[34] Current reviews summarize the present understanding about the mechanism of internalization of CPPs.[35,36] The mechanism and efficacy of cellular uptake depends also on the physicochemical properties of the cargo attached and on the cell line studied. Delivery of different type of cargos by CPPs was reviewed recently by Hudecz et al.[37] and Foged et al.[38] Interestingly, there are a few examples for the application of oligoarginines for delivery of small organic compounds. Taxol was conjugated to modified octaarginines by thioether linkage and the pH dependence of the release of the free drug from the conjugates having different linker moieties were analyzed.[39] Adenosine derivatives coupled with oligoarginine were investigated for their protein kinase inhibition and cellular uptake properties in vitro.[40] Ferrocene-derivatives as well as Dau were attached by our group to oligoarginines with amide bond and the cytostatic effect of the conjugates was evaluated in vitro.[28,41] Conjugates were also prepared for delivery of peptide/protein based drugs, like cyclosporin A, insulin, Smac (second mitochondria-derived activator of caspases) peptide and their biological activity was reported in vitro and in vivo.[42–44]

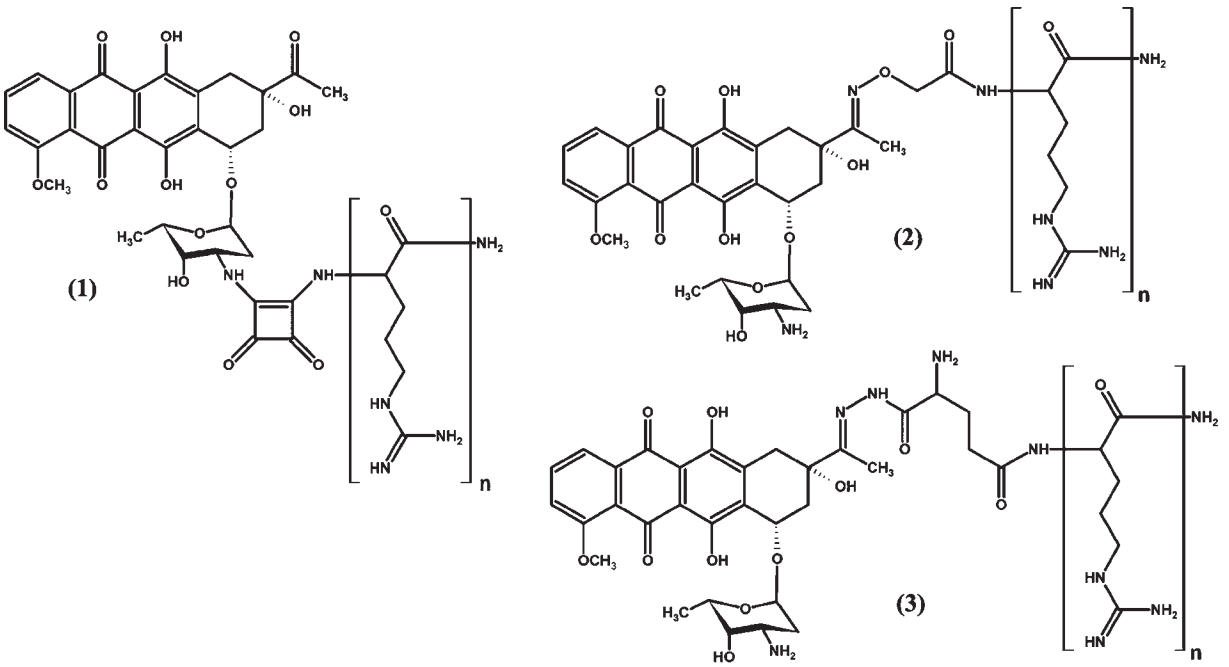


FIGURE 2 Structure of daunomycin-oligoarginine conjugates ($n = 6, 8$). Conjugate with squaric acid diamide (1); with oxime (2); and hydrazone (3) linkage.

In this article, we describe the synthesis and characteristics of a new class of Dau-conjugates in which oligoarginine with 6 or 8 residues (Arg_n, $n = 6, 8$) is attached to the drug by squaric amide, oxime, or hydrazone bond (Figure 2). Our aim was to develop appropriate coupling strategies and to study chemical (stability, spectral characteristics) and biological (cytostasis, cellular uptake) properties of Dau-conjugates as a function of covalent linkage, site of conjugation, and oligoarginine length.

According to our results, Dau-conjugates with squaric amide (Dau-□-Arg_6) or oxime (Dau=N—O—CH_2—CO—Arg_6) linkage were stable, but continuous release of free Dau was observed from the hydrazone conjugate (H—Glu(Arg_6)—NH—N=Dau) under conditions studied. The nature of chemical bond (amide, oxime or hydrazone) between Dau and the peptide has essentially no influence on the absorption spectra of chromophor in Dau-conjugates, while the amplitude of the emission spectra depends on the coupling site utilized for conjugation (amino vs. oxo function). Similarly, the cytostatic effect of Dau-conjugates was sensitive for the site of conjugation. Both cytostatic and uptake properties are influenced by the acid stability of the linkage (hydrazone vs. oxime/amide) as well as the studied cell line (HL-60 vs. HepG2).

MATERIALS AND METHODS

General Materials

Daunomycin hydrochloride was a gift from the Institute of Drug Research (IVAX, Budapest, Hungary); Fmoc-Rink-Amide MBHA resin (0.72 mmol/g) was purchased from Novabiochem (Laufelfingen, Switzerland). All amino acid derivatives were from Bachem (Bubendorf, Switzerland) or Reanal (Budapest, Hungary). Scavengers (thioanisole, ethanedithiol [EDT], phenol), coupling agents (*N,N′*-diisopropylcarbodiimide [DIC], *N,N′*-dicyclohexylcarbodiimide [DCC], 1-hydroxybenzotriazole [HOBt], *N,N′*-diisopropylethylamine [DIEA], *N*-methylmorpholine [NMM]) and cleavage reagents (piperidine, 1,8-diazabicyclo[5.4.0]undec-7-ene [DBU], trifluoroacetic acid [TFA]) were Fluka (Buchs, Switzerland) products. All solvents: acetic acid [AcOH], dimethylformamide [DMF], dimethylsulphoxide [DMSO], dichloromethane [DCM], diethyl ether, acetonitrile [ACN], trifluoroacetic acid [TFA], and ammonium acetate [NH_4OAc] were from Reanal (Budapest, Hungary). 3,4-Dimethoxy-3-cyclobutene-1,2-dione [squaric acid dimethyl ester], 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide [MTT], RPMI-1640 medium and fetal calf serum [FCS] were obtained from Sigma-Aldrich (Budapest, Hungary). HPMI (D-glucose, $NaHCO_3$, NaCl, HEPES, KCl, $MgCl_2$, $CaCl_2$, $Na_2HPO_4 \times 2H_2O$) was prepared in our laboratory using ingredients from Sigma-Aldrich (Budapest, Hungary). MCI GEL CHP20P was purchased from Supelco (Bellefonte, PA) Sephadex gels (G10 superfine, G25 superfine) were obtained from Pharmacia Fine Chemicals AB (Uppsala, Sweden).

Synthesis and Purification

Oligoarginines (Arg_n). The oligoarginine peptides (Arg_n, $n = 5, 6, 7, 8$) were synthesized on solid phase by published procedure[41] using Fmoc strategy on Fmoc-Rink-Amide MBHA resin (0.72 mmol/g). The side chain of Arg residue was protected by 2,2,4,6,7-pentamethyl-benzofuran-5-sulfonyl (Pbf) group. The coupling was performed using DIC/HOBt in situ active ester methodology in

DMF, coupling time was 90 min. The efficiency of the coupling was monitored by ninhydrine reaction.[45] The peptides were cleaved from the resin with TFA in the presence of scavengers (TFA: water: thioanizole: EDT: crystalline phenol = 10 ml: 0.5 ml: 0.5 ml: 0.25 ml: 0.75 g). The crude product was purified by preparative FPLC system (Pharmacia, Uppsala, Sweden) on a Vydac (C18, 300 Å, 25 μm, 480 × 25 mm, Vydac, Hesperia, CA) column. A linear gradient elution was developed from 0 to 20% eluent B from 0 to 40 min. Eluent A was 0.1% TFA in water, while eluent B contained 0.1% TFA in ACN-water (80:20 v/v%). Experiments were carried out at a flow rate of 8 ml/min at room temperature. Samples were dissolved in eluent A, sample concentration was 5 mg/ml and 15 ml of solution was injected. Peaks were detected at $\lambda = 220$ nm.

NH_2—O—CH_2—CO—Arg_n, (n = 6, 8, Aminooxy-Oligoarginines). Boc-aminooxy-acetic acid was coupled on solid phase to the free *N*-terminal amino group of oligoarginine (Arg_n, $n = 6, 8$) using DIC/HOBt coupling agents. Coupling time was 60 min. Peptides were cleaved from the resin as described earlier. Crude products were purified by semi-preparative RP-HPLC (Knauer, Bad Homburg, Germany) on a Phenomenex Jupiter (C18, 300 Å, 10 μm, 250 × 10 mm, Phenomenex, CA) column. Linear gradient elution was developed from 1% to 25% eluent B from 5 to 29 min. Eluent A was 0.1% TFA in water, while eluent B contained 0.1% TFA in methanol-water (90:10 v/v%). Each purification was carried out at a flow rate of 4 ml/min at room temperature. Samples were dissolved in eluent A, sample concentration was 5 mg/ml and 2 ml of solution was injected. Peaks were detected at $\lambda = 220$ nm.

Fmoc-Glu-OMe. Fmoc-Glu(O^tBu)-OMe was prepared by methylation of Fmoc-Glu(O^tBu)-OH using diazomethane in diethyl ether according to published procedures.[46] The product was crystallized from ethyl acetate and petrol ether with 87% yield (m.p.: 75–77°C; Elemental composition. Calcd. for $C_{25}H_{29}NO_6$: C, 68.32; H, 6.65; N, 3.19. Found: C, 67.66; H, 6.40; N, 3.25). Fmoc-Glu-OMe was prepared by the removal of the O^tBu-group of Fmoc-Glu(O^tBu)-OMe in 95 v/v% TFA/water. After removing TFA, the product was crystallized from ethyl acetate and petrol ether with 96% yield (m.p.: 130–132°C; Elemental composition Calcd. for $C_{21}H_{21}NO_6$: C, 65.79; H, 5.52; N, 3.65. Found: C, 65.04; H, 5.23; N, 3.68.). The overall yield was 83%.

H—Glu(Arg_n)—NH—NH_2, (n = 6, 8, Hydrazide-Oligoarginines). N^{α}-Fmoc-Glu-OMe was coupled on solid phase through its free γ-carboxylic group to the free *N*-terminal amino group of oligoarginine (Arg_n, $n = 6, 8$) using DIC/HOBt coupling agents. Coupling time was 60 min. Then the α-methyl ester-group of the Glu residue was reacted with hydrazine hydrate in DMF to form hydrazide bond. Finally, the N^{α}-Fmoc-group was removed from the Glu residue by 2 v/v% piperidine, 2 v/v% DBU in DMF. The peptides were cleaved from the resin and purified by FPLC as well as the oligoarginine peptides.

Daunomycinyl-Arginyl-Squaric Acid Diamide (Dau-□-Arg). Daunomycinyl-squaric acid monoamide (Dau-□-OMe) was synthesized according to published procedures[20] by the reaction of Dau hydrochloride with squaric acid dimethyl ester in methanol. Briefly, 45 mg (0.08 mmol) Dau hydrochloride was dissolved in 3 ml MeOH then 20 μl (0.11 mmol) DIEA was added to the solution and stirred for 10 min; 13.3 mg (0.09 mmol, 12% molar excess) squaric acid dimehtyl ester was dissolved separately in 2 ml MeOH and the mixture was added to the Dau solution. Further 10 μl (0.06 mmol) DIEA was added to the reaction mixture to adjust the pH to 7. The mixture was stirred at room temperature. The reaction was followed by analytical RP-HPLC. The product was formed in 5 h. The reaction was carried on without isolation of daunomycinyl-squaric acid monoamide. The pH was adjusted to 9 by 30 μl (0.18 mmol) DIEA, and 23 mg (0.13 mmol, 62% molar excess) L-Arg was added to the reaction mixture in 0.5 ml methanol–water (50:50 v/v%) mixed solvent. The mixture was stirred at room temperature. Red crystals were precipitated from the solvent within 1 day. The crystals were filtered, washed with water, and dried in vacuum. The remaining reaction mixture was also purified on G10 Sephadex gel with 0.1*M* NH_4OAc as eluent. The red product could be visualized. After removing the solvent, Dau-□-Arg was dissolved in AcOH-water (10:90 v/v%) and liophylized.

Daunomycinyl-Oligoarginyl-Squaric Acid Diamide (Dau-□-Arg_n, n = 6, 8).

Method 1. The reaction between Dau-□-OMe and oligoarginine peptides (Arg_n, $n = 6, 8$) was performed in methanol-water (77:23 v/v%) mixed solvent as described earlier.[47] The pH was set to 9 by DIEA. 6.5 mg (0.01 mmol) Dau-□-OMe was solved in 3.5 ml MeOH then 50 μl (0.32 mmol) DIEA and 0.03 mmol (200% molar excess) oligoarginine in 1.0 ml water were added. The reaction was monitored by analytical RP-HPLC. No appropriate product formation was observed under these circumstances.

Method 2. The reaction between Dau-□-Arg and oligoarginine peptides (Arg_n, $n = 5, 7$) was performed in DMSO using DCC and HOBt coupling agents; 11 mg (0.014 mmol) Dau-□-Arg, 2.6 mg (0.017 mmol, 20% molar excess) HOBt and 20 mg (0.097 mmol, ~ 600% molar excess) DCC were solved in 200 μl DMSO then 0.021 mmol (50% molar excess) peptide was solved in 200 μl DMSO and 10 μl (0.09 mmmol) NMM was added to the solution to maintain the required pH (pH 6–7). The two solutions were mixed and stirred at room temperature. The reaction was followed by analytical RP-HPLC, the product was formed in a couple of hours. The target compound was purified by gel-filtration on G25 Sephadex with 0.1*M* NH_4OAc as eluent. The red products could be visualized. The conjugates were further purified by MCI gel chromatography to remove the NH_4OAc salt as well as the free peptide. For this, the following protocol was used: the gel was washed with methanol (3 × 5 min) then with distilled water (3 × 5 min) the conjugate was added to the gel in diluted solution, the solvent was removed, the gel was washed with distilled water, and the conjugate was eluated from the gel by methanol-water (80:20 v/v%). After the removal of methanol in vacuum, the conjugate sample was dissolved in distilled water and liophylized.

Oxime Conjugate of Daunomycin With Oligoarginine (Dau=N—O—CH_2—CO—Arg_n, n = 6, 8). Dau hydrochloride (0.018 mmol) and NH_2—O—CH_2—CO—Arg_n ($n = 6, 8$) peptide (0.022 mmol, 20% molar excess) were dissolved in 0.1*M* NaOAc/AcOH buffer (pH = 5.2) and DMSO (50:50 v/v%) mixed solvent. The mixture was stirred at room temperature. The reaction was monitored by analytical RP-HPLC, the product was formed in 1

day. The crude product was purified by gel-filtration on G25 Sephadex and on MCI gel. The purified conjugate was dissolved in distilled water and freeze-dried.

Hydrazone Conjugates of Daunomycin With Oligoarginines (H—Glu(Arg$_n$)—NH—N=Dau, n = 6, 8). Dau hydrochloride (0.018 mmol) and H—Glu(Arg$_n$)—NH—NH$_2$ (n = 6, 8) peptide (0.022 mmol) were dissolved in the mixture (50:50 v/v%) of 0.1*M* NaOAc/AcOH buffer (pH = 5.8) and DMF. The mixture was stirred at room temperature. The reaction was followed by analytical RP-HPLC, the product was formed in one day. The crude product, as well as the squaric acid and oxime conjugates, was purified by gel-filtration on G25 Sephadex and on MCI gel. The purified conjugate was dissolved in distilled water and freeze-dried.

Characterization of Compounds

Analytical RP-HPLC. All peptides, Dau-□-Arg$_n$ (n = 1, 6, 8) and Dau=N—O—CH$_2$—CO—Arg$_m$ (m = 6, 8) conjugates were analyzed by Knauer RP-HPLC system (Bad Homburg, Germany). Each purification was performed by RP-HPLC using Supleco Supelcosil^{TM} LC-18-DB (C18, 120 Å, 5 μm, 4.6 × 250 mm; Bellefonte, PA) column. A linear gradient elution was developed from 5% to 95% eluent B from 5 to 50 min. Eluent A was 0.1% TFA in water, while eluent B contained 0.1% TFA in ACN-water (80:20 v/v%). Experiments were carried out at a flow rate of 1 ml/min at room temperature. Samples were dissolved in water and 20 μl of solution was injected. Peaks were detected at λ = 220 nm.

Analytical RP-HPLC of Hydrazone Conjugates. Monitoring of the reactions in which hydrazone conjugates were formed was controlled by RP-HPLC using a Knauer (Bad Homburg, Germany) system. The measurements were performed using Hamilton PRP-X100 anion exchange column (10 μm, 4.6 × 250 mm; Hamilton, Reno, NV). After the reaction, the purified product was analysed using the same system. A linear gradient elution was developed: 0% eluent B from 0 to 3 min, 0% to 100% eluent B from 3 to 13 min, 100% eluent B from 13 to 20 min. Eluent A was 0.1*M* NH_4OAc in water, while eluent B was 0.1*M* NH_4OAc in methanol-water (90:10 v/v%). Each purification was carried out at a flow rate of 1.5 ml/min at room temperature. Samples were dissolved in 0.1*M* NH_4OAc in water and 20 μl of solution was injected. Peaks were detected at λ = 490 or λ = 220 nm.

Mass Spectrometry. The identification of oligoarginine peptides was achieved by electrospray ionization mass spectrometry (ESI–MS) on a Bruker Daltonics Esquire 3000 Plus (Bremen, Germany) ion trap mass spectrometer, operating with continuous sample injection at 4 μl/min flow rate. Samples were dissolved in ACN–water (50:50 v/v%) mixture containing 0.1 v/v% AcOH. Mass spectra were recorded in positive ion mode in the m/z 200–2000 range.

Mass spectrometric analysis of the conjugates was performed using a ProteinChip Surface-Enhanced Laser Desorption/Ionization Time-of-Flight (SELDI-TOF) system equipped with a nitrogen laser and a linear TOF analyzer (PS4000 Enterprise Edition, Bio-Rad Laboratories). Samples were dissolved in ACN–water (50:50 v/v%) mixture. For the analysis, the following procedure was used: the surface of a Surface Enhanced Neat Desorption (SEND) ID type ProteinChip array was washed with 5 μl 0.1% TFA for 10 s, then the solvent was removed and the spots were dried on air; 1 μl sample was added to the surface, and dried in air. The arrays were analyzed in the 300–5000 m/z range in positive ion mode.

Stability Assay. Stability of hexaarginine conjugates (Dau-□-Arg$_6$, Dau=N—O—CH$_2$—CO—Arg$_6$, H—Glu(Arg$_6$)—NH.—N=Dau) was investigated as selected representatives in distilled water and at pH = 5 and pH = 2 in 0.1*M* Na-citrate/citric acid buffer. Dau was used as control compound. Samples from the solutions of Dau-□-Arg$_6$ and Dau=N—O—CH$_2$—CO—Arg$_6$ conjugates as well as Dau were taken at 5 min, 1 and 24 h, 1 week and 2 weeks and were analyzed by RP-HPLC as described earlier. Samples from the solutions of H—Glu(Arg$_6$)—NH—N=Dau conjugate in pH = 2 and 5 buffers were taken at 2, 35, and 70 min, while samples from the solution of H—Glu(Arg$_6$)—NH—N=Dau in distilled water were taken at 2 and 70 min, 24 and 48 h. The homogeneity of the solid conjugates was checked regularly for 2 months. In this case, solutions were prepared freshly and the composition of the sample was studied by RP-HPLC as described earlier.

Singlet State Studies. Ground-state absorption spectra were recorded with 1 nm steps and 2 nm bandwidth using a Cary 4E (Varian, Mulgrave, Australia) spectrophotometer. Spectra of Dau and its hexaarginine conjugates were recorded at 1.8–1.9 × 10^{-5}*M* concentration in distilled water. Corrected steady-state emission (λ_{ex} = 473) and excitation (λ_{em} = 591) spectra were obtained using Fluorolog-3 (Yvon Jobin, France) spectroluminometer.

In Vitro Biological Activity

In Vitro Cytostatic Effect. HL-60 human leukemia cells (ATCC: CCL-240) and HepG2 human hepatoma cells (ATCC: HB-8065) were cultured in RPMI-1640 medium supplemented with 10% FCS, 2 m*M* L-glutamine and 160 μg/ml gentamycin.[48] Cell culture was maintained at 37°C in a humidified atmosphere with 5% CO_2. To study the cytostatic effect of the conjugates 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide-assay (MTT-assay) was carried out[49]; 5 × 10^3 cells per well were plated on 96-well plates. After 24 h incubation at 37°C, cells were treated for 3 h with the conjugates dissolved in serum-free RPMI-1640 medium. The compounds were used in the 2.6 × 10^{-4}–10^2 μ*M* concentration range. Cells treated with serum-free medium for 3 h were used for control. After treatment and incubation, cells were washed twice with serum-free medium and cultured for 72 h in complete medium. On the fourth day, MTT-assay was carried out. 45 μl MTT-solution (2 mg/ml) was added to each well (final concentration: 367 μg/ml). After 3.5 h incubation, purple crystals were formed by mitochondrial dehydrogenase enzyme of live cells. Cells were centrifuged for 5 min at 863g and supernatant was removed. Crystals were dissolved in DMSO and the optical density (OD) of the samples was measured at λ = 540 and 620 nm using ELISA Reader (Labsystems MS reader, Finland). OD_{620} was substracted from OD_{540}. The percent of cytostasis was calculated using the following equation:

$$\text{Cytostasis \%} = [1-(OD_{treated}/OD_{control})] \times 100.$$

where $OD_{treated}$ and $OD_{control}$ correspond to the optical densities of treated and control cells, respectively. Cytostasis % was plotted as a function of concentration, fitted to a sigmoidal curve, IC_{50} value was determined by right of this curve and illustrated in bar chart.

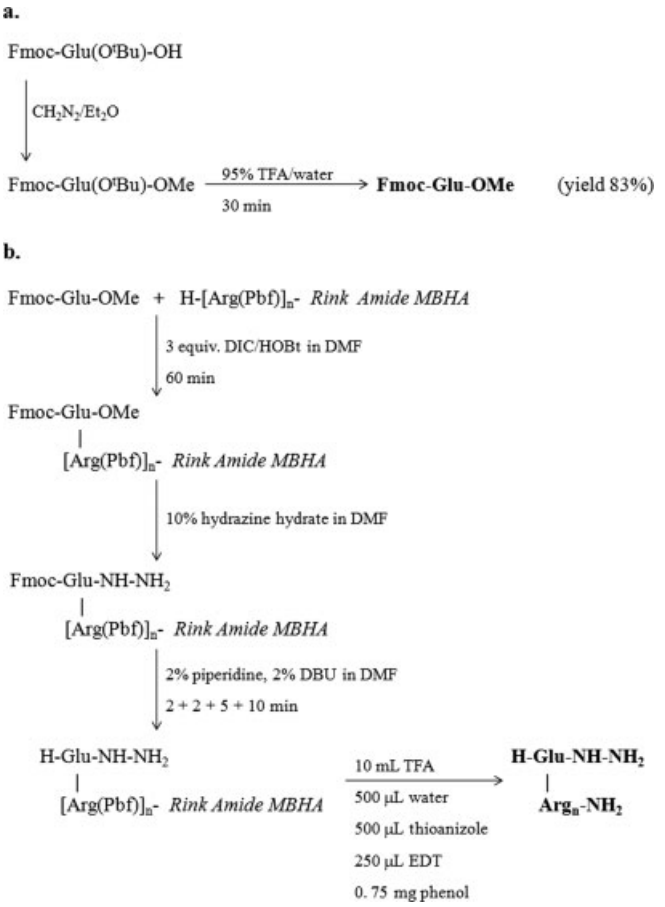
Cellular Uptake. HL-60 human leukemia and HepG2 human hepatoma cells were cultured in RPMI-1640 (Sigma, St. Louis) medium supplemented with 10% FCS, 2 m*M* L-glutamine and 160 μg/ml gentamycin. Cell culture was maintained at 37°C in a humidified atmosphere with 5% CO_2. To study the cellular uptake of the conjugates, 10^5 cells per well were plated on 24-well plates. After 24 h incubation at 37°C, cells were treated for 1.5 h with the conjugates solved in serum-free RPMI-1640 medium. The compounds were used in the 0.8–100 μM concentration range. Cells treated with serum-free medium for 1.5 h were used for control. After treatment and incubation, cells were washed with HPMI and treated with trypsin for 10 min. The effect of trypsin was stopped with HPMI supplemented with 10% FCS and cells were moved from the plate to FACS-tubes. After washing, cells were re-suspended in HPMI. Fluorescence intensity of HL-60 and HepG2 cells was monitored by flow cytometry (BD LSR II, BD Bioscience, San Jose, CA). Data were analyzed with FACSDiVa software. Fluorescence intensity was illustrated in bar chart.

RESULTS

In this study, we report on the synthesis and characteristics of a new group of Dau–oligoarginine conjugates containing different covalent linkage (squaric amide, oxime, or hydrazone bond) between the two components. The conjugates were characterized by RP-HPLC and MS. The stability of the compounds was analyzed both in solid form and in solution. Considering the importance of the fluorescence feature of daunomycin, Dau-conjugates with hexaarginine were analyzed to investigate the potential effect of the linkage between the two moieties on the fluorescence properties. To establish correlation between the structure (conjugation site, type of the covalent bond) and cytostatic effect as well as cellular uptake properties, the Dau-conjugates and the free components were studied on both HL-60 human leukemia and HepG2 human hepatoma cells.

Synthesis and Purification

Preparation of Modified Oligoarginine Peptides. For the preparation of the conjugates, *N*-terminally modified oligoarginine peptides were synthesized on solid phase by Fmoc-strategy using Fmoc-Arg(Pbf)-OH amino acid derivative. In case of NH_2—O—CH_2—CO—Arg_n and H—Glu(Arg_n)—NH—NH_2 peptides (n = 6, 8) Boc-aminooxy acetic acid or Fmoc-glutamic acid methylester—prepared from N^{α}-Fmoc-Glu(^{t}Bu)—OH with good purity and 83% yield—was coupled to the *N*-terminus of the oligoarginine peptides by the same coupling technique as outlined in Scheme 1. Reaction proceeded smoothly in both cases. It was also observed that for completeness even shorter (1 h) reaction time was needed as compared to the 1.5 h coupling time applied for Arg.



SCHEME 1 Outline of the synthesis of oligoarginine peptides containing hydrazide group (n = 6, 8): synthesis of Fmoc-Glu-OMe (a); synthesis of hydrazide-peptide (b).

After cleavage from the solid support, peptides—H—Glu(Arg_n)—NH—NH_2 as well as oligoarginine—were purified by FPLC using ACN-water (80:20 v/v%) containing 0.1% TFA as eluent B. However, we found that the aminooxy-group of NH_2—O—CH_2—CO—Arg_n peptides could react rapidly with oxo-compound(s) possibly present in the eluent forming oxime bond. Namely we have detected such derivative after the RP-HPLC purification using ACN-water (80:20 v/v%) containing 0.1% TFA as eluent B. We hypothesized the presence of a small amount of acetone in the eluent and this could react with aminooxy-oligoarginine peptides resulting in peptide derivatives unable to react with Dau during the conjugation reaction. This difficulty could be solved either by using freshly prepared eluent B or by using MeOH-water (90:10 v/v%) mixture containing 0.1% TFA as eluent B.[50] The modified and also the free oligoarginine peptides were characterized by mass spectrometry and retention time value (R_t) and obtained by RP-HPLC. The characteristics of these compounds are summarized in Table I.

Table I Characteristics of the Peptide

Peptides	MS (M)[a] Calculated	MS (M)[a] Measured	R_t (min)[b]
$H_2N—Arg_5—NH_2$	798.0	797.7	7.5
$H_2N—Arg_6—NH_2$	954.2	953.7	14.3
$H_2N—Arg_7—NH_2$	1110.3	1110.0	17.1
$H_2N—Arg_8—NH_2$	1266.5	1265.1	18.2
$H_2N—O—CH_2—CO—NH—Arg_6—NH_2$	1027.2	1026.8	16.6
$H_2N—O—CH_2—CO—NH—Arg_8—NH_2$	1339.6	1339.0	18.2
$NH_2—Glu(Arg_6)—NH—NH_2$	1097.3	1097.2	15.1
$NH_2—Glu(Arg_8)—NH—NH_2$	1409.7	1409.5	18.9

[a] ESI–MS.
[b] HPLC retention time, Supelcosil™ LC-18-DB (C18, 120 Å, 5 μm, 4.6 × 250 mm; Bellefonte, PA) column, gradient: 0–5 min 5% B eluent, 5–50 min 95% B eluent, where eluent A: 0.1% TFA in water, eluent B: 0.1% TFA in ACN–water (80:20 v/v%).

Daunomycinyl-Arginyl- and Oligoarginyl-Squaric Acid Diamide (Dau-□-Arg, Dau-□-Arg$_n$, n = 6, 8). Dau-□-Arg was synthesized from Dau, squaric acid dimethyl ester, and L-Arg in a two-step procedure[47] with some modifications. Without purification between the two reaction steps, higher reaction yield could be achieved.

Two methods were developed and investigated for the preparation of daunomycinyl-oligoarginyl-squaric acid diamide (Dau-□-Arg$_n$, $n = 6, 8$) conjugates. First Dau-□-OMe and oligoarginine peptides (Arg$_n$, $n = 6, 8$) were dissolved in methanol-water mixture (Method 1) under those conditions, which were used for the preparation of Dau-□-Arg from Dau-□-OMe and L-Arg. Even the elongation of the reaction time was not resulting in the desired Dau-□-Arg$_n$ conjugates. Because of the limited solubility of oligoarginines unlike L-Arg in methanol, water was added to the reaction mixture. This led to the formation of inactive side product of squaric acid monoamide, which is no longer able to react with oligoarginines. Therefore, another strategy was developed. In this case (Method 2), Dau-□-Arg and oligoarginine peptides (Arg$_n$, $n = 5, 7$) were reacted applying an active ester coupling method, based on DCC and HOBt. To compensate the effect of TFA which can remain attached to the oligoarginine peptides after purification and freeze-drying, NMM was used to maintain the pH value between 6 and 7. The reaction proceeded fast and according to the HPLC analysis the products were formed in a couple of hours. With this new strategy, preparation of squaric acid conjugates of other large and/or hydrophilic peptides could be carried out.

Oxime and Hydrazone Conjugates of Daunomycin With Oligoarginines (Dau=N—O—CH$_2$—CO—Arg$_n$ and H—Glu(Arg$_n$)—NH—N=Dau, n = 6, 8). These two types of conjugates were prepared in similar reactions. Aminooxy- as well as hydrazide-group reacts spontaneously with aldehydes and ketones in aqueous solvent even under mild acidic pH. The 14-oxo-group of Dau reacted with the aminooxy- and hydrazide-group. No side-product formation was observed and the target compounds were formed in 1 day.

Purification of Dau Conjugates. All of the Dau–oligoarginine conjugates were purified by gel filtration on G25 Sephadex gel and by MCI gel chromatography. During purification, the aim was to avoid the use of TFA which is a common additive in RP-HPLC eluents for peptide purification. Dau is an acid-sensitive compound and while its analysis by RP-HPLC is possible with TFA containing eluents, a long-time purification with the same method can cause some damage. TFA can form a salt with guanidino group on the side chains of arginine residues or on the amino terminus of the peptide because of that side-products could appear even in the lyophilized product.

During gel filtration, the eluent was 0.1M NH_4OAc in water. After gel filtration, the NH_4OAc and the remained peptide were removed by MCI gel chromatography. During this step, all water-soluble components with aromatic moiety are retained by MCI gel. After washing out the impurities (e.g., unconjugated oligoarginine peptides) with water, the Dau-conjugates possessing anthracycline ring were eluted from the gel by organic solvents as described in the experimental section.

Characterization of the Compounds

Homogeneity of the Conjugates. Homogeneity of the conjugates was investigated by chromatography as well as by mass spectrometry. In most cases, RP-HPLC measurement

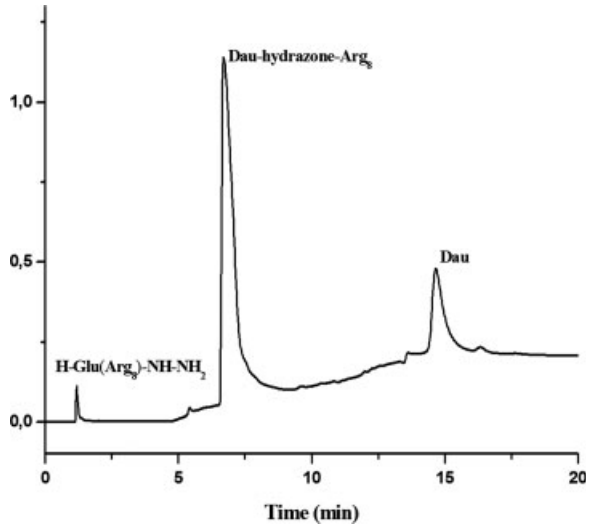


FIGURE 3 Analytical HPLC chromatogram of H—Glu(Arg_8)—NH—NH_2 peptide, H—Glu(Arg_8)—NH—N=Dau conjugate, and free daunomycin. Hamilton PRP-X100 anion exchange (10 μm, 4.6 × 250 mm; Hamilton, Reno, NV) column. Gradient elution: 0% eluent B from 0 to 3 min, 0% to 100% eluent B from 3 to 13 min, 100% eluent B from 13 to 20 min, where eluent A: 0.1*M* NH_4OAc in water, eluent B: 0.1*M* NH_4OAc in MeOH-water (90:10 v/v%). Flow rate: 1.5 ml/min at room temperature; 20 μl of samples in 0.1*M* NH_4OAc/water were injected and peaks were detected at λ = 220 nm.

was performed on C18 column with TFA containing eluents. In case of conjugates with strongly acid-sensitive hydrazone linkage (H—Glu(Arg_n)—NH—N=Dau), this type of analysis was not appropriate. We observed that after the injection of hydrazone conjugate only Dau was eluted. This was due to the presence of TFA in the eluents. Therefore, a new HPLC method was developed by the utilization of Hamilton PRP-X100 anion exchange column. The eluents were 0.1*M* NH_4OAc in water and 0.1*M* NH_4OAc in methanol-water (90:10 v/v%). By this methodology, efficient separation of hydrazide-peptide, the Dau-conjugate (H—Glu(Arg_8)—NH—N=Dau), and free Dau was achieved as demonstrated in Figure 3. By this way, it was possible to analyze the homogeneity of conjugates even with highly acid-sensitive hydrazone linkage and to prove that the samples do not contain free Dau.

Peptides were also analyzed by electrospray ionization mass spectrometry. Because of the high number of arginine residues in the conjugates, predominantly multiplied protonated molecular ions were detected in the mass spectra (ions with two, three, or four protons). Charging of the oligoarginine peptides was usually high, resulting in intensive ions in surprisingly low mass range (200–500 *m/z*). Analysis of the Dau-conjugates by electrospray ionization mass spectrometry resulted in highly complex mass spectra, showing high charging of the molecules and rapid fragmentation. Therefore, conjugates were further analyzed by SELDI-TOF mass spectrometry. In this case, a ProteinChip array containing a surface integrated matrix compound was used (Surface Enhanced Neat Desorption, SEND) to reduce the number of interfering matrix peaks in the low mass range and to minimize the contact of the acid-sensitive compounds with acidic solvent or matrix. In this way, intensive singly protonated molecular ions of the Dau-conjugates were detected in the SELDI mass spectra. The characteristics of the conjugates studied are summarized in Table II.

Stability. The stability of three Dau-hexaarginine conjugates Dau-□-Arg_6, Dau=N—O—CH_2—CO—Arg_6, H—Glu(Arg_6)—NH—N=Dau) as selected representative compounds was investigated in distilled water as well as in 0.1*M* Na-citrate/citric acid buffer at pH = 5 and pH = 2. Dau was also included as control compound. Dau-conjugates with squaric amide and oxime linkage as well as Dau proved to be stable under these circumstances at least for 2 weeks. In contrast, continuous release of free Dau was observed from the hydrazone conjugate (H—Glu(Arg_6)—NH—N=Dau) as shown in Figure 4. In 0.1*M* Na-citrate/citric acid buffer at pH = 2, the half-life time of this conjugate was 25 min, while at pH = 5 and also in distilled water this compound was slightly more stable ($t_{1/2}$ = 70 min and $t_{1/2}$ = 48 h, respectively).

Dau-hexaarginine conjugates with squaric amide Dau-□-Arg_6) or oxime (Dau=N—O—CH_2—CO—Arg_6) bond kept at 4°C as well as H—Glu(Arg_n)—NH—N=Dau kept at −20°C in solid form proved to be stable.

Spectroscopic Properties. The ground-state absorption properties of three Dau-hexaarginine conjugates Dau-□-Arg_6, Dau=N—O—CH_2—CO—Arg_6, H—Glu(Arg_6)—NH—N=Dau) were studied in distilled water. The recorded spectra are typical of Dau (data not shown),[51] consisting of the large complex absorption band between λ = 400 and λ = 570 nm. The main peak of the spectrum is at λ = 500 nm, the secondary one is around λ = 483 nm. Inspection of the absorption spectra of Dau conjugates reveals that the spectrum of the chromophore is essentially not influenced by the presence of the hexapeptide or by the chemical bond (amide, oxime, or hydrazone bond) between Dau and the peptide.

The cellular accumulation of Dau and its conjugates can be followed by the fluorescent signal related to the drug moiety.[52,53] For the better understanding of these signals, the comparison of the fluorescence spectra of free Dau and its hexaarginine conjugates is necessary. The corrected emission

Table II Characteristics of Dau–Peptide Conjugates and Dau

	MS (M)[a]			IC_{50} (μM)[c]		Cellular uptake[d]	
Compounds	Calculated	Measured	R_t (min)[b]	HL-60	HepG2	HL-60	HepG2
Dau-□-Arg	779.7	780.5	35.6	n.d.	n.d.	n.d.	n.d.
Dau-□-Arg_6	1559.6	1559.5	32.1	5.16	28.74	3664	325
Dau-□-Arg_8	1872.0	1872.0	32.0	15.49	58.41	2629	324
Dau=N—O—CH_2—CO—NH—Arg_6	1536.7	1536.3	26.5	3.02	22.87	4391	259
Dau=N—O—CH_2—CO—NH—Arg_8	1849.1	1848.6	26.1	5.57	27.11	2716	579
NH_2—Glu(Arg_6)—NH—N=Dau	1606.8	1607.6	7.3[e]	8.41	6.62	1868	250
NH_2—Glu(Arg_8)—NH—N=Dau	1919.2	1919.2	6.7[e]	2.59	4.74	3775	388
Daunomycin	527.5	n.d.	34.9	0.05	0.63	48621	10131

[a] SELDI–MS.

[b] HPLC retention time, Column: Supelcosil™ LC-18-DB (C18, 120 Å, 5 μm, 4.6 × 250 mm; Bellefonte, PA), gradient elution: 0–5 min 5% eluent B, 5–50 min 90% eluent B, where eluent A: 0.1% TFA in water, eluent B: 0.1% TFA in acetonitrile–water (80:20 v/v%).

[c] IC_{50} values in μM determined as described in Materials and Methods section.

[d] Fluorescence intensity measured after treatment of the cells at $c = 20$ μM as described in Materials and Methods section.

[e] HPLC retention time, Column: HAMILTON PRP-X100 anion exchange (10 μm, 4.6 × 250 mm; Hamilton, Reno, NV), gradient elution: 0–3 min 0% eluent B, 3–13 min 100% eluent B, 13–20 min 100% eluent B where eluent A: 100 mM NH_4OAc in water, eluent B: 100 mM NH_4OAc in methanol–water (90:10 v/v%).

spectra of Dau and its hexaarginine conjugates Dau-□-Arg_6, Dau=N—O—CH_2—CO—Arg_6, H—Glu(Arg_6)—NH—N=Dau) recorded upon excitation at $\lambda = 473$ nm are shown In Figure 5.

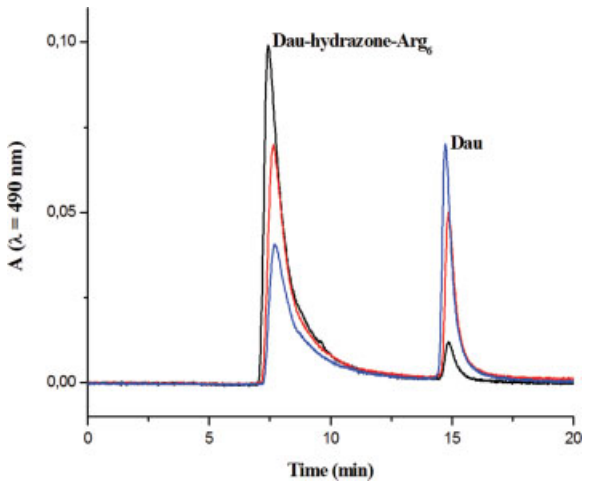


FIGURE 4 Time dependence of the release of free daunomycin from H—Glu(Arg_6)—NH—N=Dau conjugate. RP-HPLC chromatograms were recorded in 0.1M Na-citrate/citric acid buffer, pH = 5. Sample was dissolved and injected after 2 min (black), 35 min (red), or 70 min (blue) as described in the Materials and Methods section. Hamilton PRP-X100 anion exchange (10 μm, 4.6 × 250 mm; Hamilton, Reno, NV) column. Gradient: 0% eluent B from 0 to 3 min, 0 to 100% eluent B from 3 to 13 min, 100% eluent B from 13 to 20 min, where eluent A: 0.1M NH_4OAc in water, eluent B: 0.1M NH_4OAc in MeOH-water (90:10 v/v%). Flow rate: 1.5 ml/min at room temperature; 20 μl of samples in 0.1M NH_4OAc/water were injected and peaks were detected at $\lambda = 490$.

In all cases, the main peak of the spectrum is at $\lambda = 593$ nm, the secondary peak is centered around $\lambda = 558$ nm. As one can see, the shape of the curves is insensitive to the presence of hexaarginine in the conjugate or to the chemical bond between Dau and hexapeptide, while the amplitude of the spectra depends on the coupling site utilized for conjugation (amino group or oxo group, see Figure 1). Compounds conjugated via the oxo group of Dau exhibit almost identical patterns, which are very similar to that of the free drug.

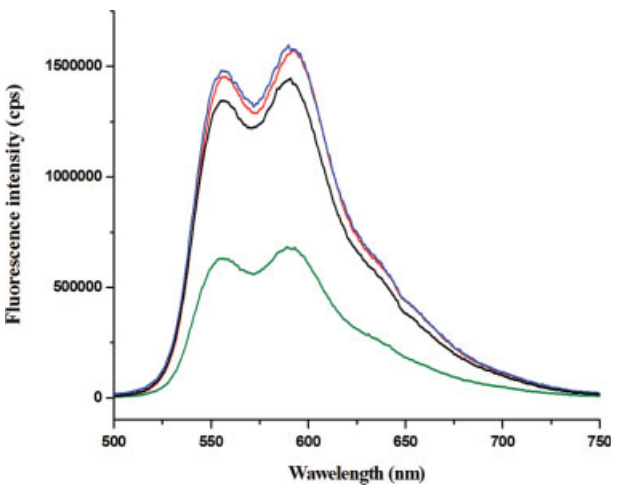


FIGURE 5 The effect of covalent linkage on the emission spectra of daunomycin-hexaarginine conjugates. Spectra were recorded in distilled water upon excitation at $\lambda = 473$ nm: Dau-□-Arg_6 (green), Dau=N—O—CH_2—CO—Arg_6 (blue), H—Glu(Arg_6)—NH—N=Dau (red) and Dau (black).

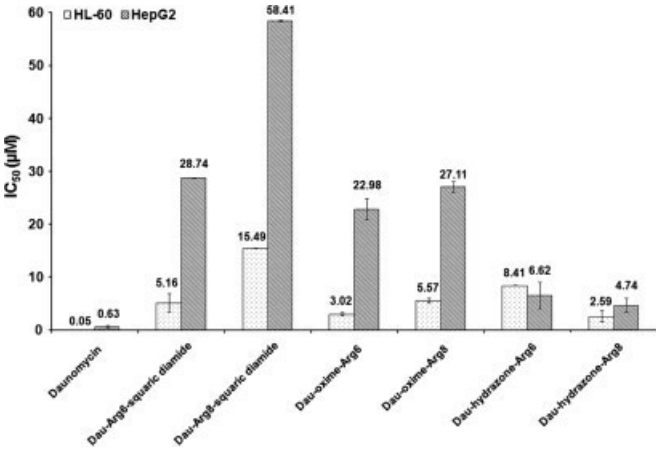


FIGURE 6 The effect of the Dau-oligoarginine conjugates on the viability of HL-60 and HepG2 cells. Bars represent $IC_{50} \pm SD$ values after the 3-h treatment. Dau was used as control.

In contrast, the amplitude of the spectrum belonging to Dau-□-Arg_6 markedly differs from the above described curves.

In Vitro Biological Activity

The cytostatic effect as well as the cellular uptake properties of Dau–oligoarginine conjugates were studied and compared with the free drug in vitro. Therefor HL-60 human leukemia and HepG2 human hepatoma cells were treated with compounds at various concentrations in the range of 0.8–100 *μM*. Cytostatic effect of the compounds was determined by MTT-assay and characterized by the IC_{50} values. Data are presented in Figure 6 and Table II. On the basis of the mean fluorescence intensity of cells before and after treatment, the uptake of the compounds was measured with flow cytometry. Data are summarized in Figures 7 and 8 and in Table II.

Cytostatic Effect. On the basis of the data outlined in Figure 6 and summarized in Table II, the effect of structural elements (type of the linkage between the two components, length of the Arg peptide) of the conjugates could be described on HL-60 cells. Octaarginine conjugates containing squaric acid linker Dau-□-Arg_8) was markedly less cytostatic than compounds with oxime or hydrazone bond (Dau=N—O—CH_2—CO—Arg_8 and H—Glu(Arg_8)—NH—N=Dau). Interestingly, when Arg_6 is present, the cytostatic effect of all three conjugates is rather similar on HL-60 cells (IC_{50} values are in the range of 3.0–8.4, $IC_{50} = 5.16$ for Dau-□-Arg_6, $IC_{50} = 3.02$ for Dau=N—O—CH_2—CO—Arg_6, and $IC_{50} = 8.41$ for H—Glu(Arg_6)—NH—N=Dau).

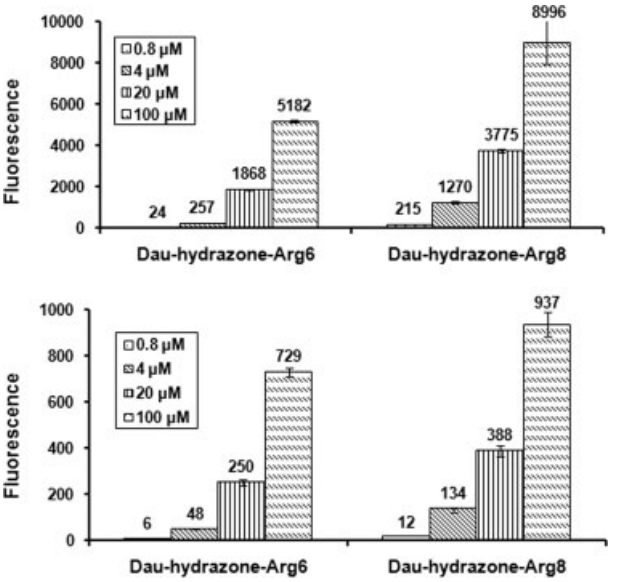


FIGURE 7 Concentration dependence of the uptake of Dau-hydrazone conjugates (H—Glu(Arg_n)—NH—N=Dau) by HL-60 (a) and HepG2 (b) cells after 1.5 h incubation with the compounds. Symbols represent the average of mean fluorescence intensity values of a representative experiment of two independent assays ± SD after subtracting the control.

Concerning the effect of length of the Arg peptide in the conjugate in all three linkage-group, different tendency could be observed. In the squaric acid linkage group, Dau-□-Arg_6 was more efficient than Dau-□-Arg_8. In contrast, in the hydrazone linkage-group, the presence of 8 Arg residues provides more cytostatic variant (H—Glu(Arg_8)—NH—N=Dau > H—Glu(Arg_6)—NH—N=Dau), while in the oxime linkage-group cytostatic effect of hexa- and octa-arginine conjugates was similar (Dau=N—O—CH_2—CO—Arg_6 ~ Dau=N—O—CH_2—CO—Arg_8).

It is important to note that very similar findings were observed on the cytostatic effect of conjugates in case of

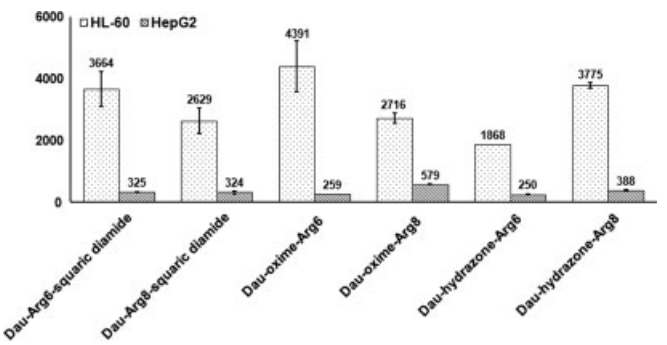


FIGURE 8 Uptake of Dau-oligoarginine conjugates by HL-60 or HepG2 cells at $c = 20$ *μM*. Columns represent the average of mean fluorescence intensity values of a representative of two independent assays ± SD after subtracting the control. Cells were treated for 1.5 h.

HepG2 hepatoma cells. Conjugates containing squaric acid linker Dau-□-Arg_n) are less cytostatic than compounds with oxime or hydrazone bond (Dau=N—O—CH_2—CO—Arg_n and H—Glu(Arg_n)—NH—N=Dau). However, in this case, the oxime-linkage goup (Dau=N—O—CH_2—CO—Arg_n) is less cytostatic than the conjugates with hydrazone bond (H—Glu(Arg_n)—NH—N=Dau) regardless of the number of presented Arg residue. Tendencies mentioned above considering the number of Arg residues within a linkage group are rather similar to those documented for HL-60 leukemia cells. As expected, free Dau was highly cytostatic on both cell types (Table II) and oligoarginines had no such effect even at the elevated concentration (data not shown).

It is worth to mention that on both cell types, the hydrazone linkage-group of Dau octaarginine conjugates (H—Glu(Arg_8)—NH—N=Dau) were highly and similarly cytostatic.

Cellular Uptake. Cellular uptake properties of compounds were studied by utilizing the fluorescence feature of Dau. As mentioned earlier, the emission spectra of the chromophore is very similar to the hexaarginine conjugates regardless of the nature of the chemical bond (amide, oxime, or hydrazone bond) between Dau and the peptide. However, the fluorescence spectra of Dau-hexaargininine conjugates and of the free drug indicate some differences between the Dau-conjugates. We found, that conjugates with oxime (Dau=N—O—CH_2—CO—Arg_6) or hydrazone (H—Glu(Arg_6)—NH—N=Dau) linkage and the free Dau display almost identical fluorescence spectral patterns (Figure 5). The amplitude of the spectrum of conjugate with squaric acid linker Dau-□-Arg_6) differs markedly.

Cellular uptake data expressed as mean fluorescence intensity are presented in Figures 7 and 8 and in Table II. These Figures were used to identify tendencies related to the traffic of Dau-conjugates into cells. Considering the differences in fluorescence properties of conjugates mentioned earlier, we should handle the potentially proportional relationship between mean fluorescence intensity measured by FACS and cellular uptake of the compounds with caution.

The uptake of the compounds proved to be concentration dependent at all concentrations studied in the 0.8–100 μM range in case of both HL-60 and HepG2 cells as demonstrated with hydrazone linkage group conjugate (H—Glu(Arg_n)—NH—N=Dau) in Figure 7.

For comparative analysis, data obtained after incubation of cells with compounds at $c = 20$ μM are shown (Figure 8 and Table II). Following the method used for the analysis of cytostatic effect, first we describe the effect of structural elements (type of the linkage, length of the Arg peptide) of the conjugates on the uptake by HL-60 cells. Data outlined in Figure 8 and in Table II showed that Dau-hexaarginine conjugates containing squaric acid linker (Dau-□-Arg_6) or oxime linkage (Dau=N—O—CH_2—CO—Arg_6) are taken up more efficiently than compound with hydrazone bond (H—Glu(Arg_6)—NH—N=Dau,). Interestingly, slightly reversed observation could be made when Arg_8 is presented: the translocation of the two conjugates Dau-□-Arg_8 and Dau=N—O—CH_2—CO—Arg_8 is less pronounced than that of H—Glu(Arg_8)—NH—N=Dau (Figure 8).

In the squaric amide group, the cellular uptake of Dau-□-Arg_6 was higher compared with Dau-□-Arg_8. The same pattern could be appreciated within the oxime linkage group (Dau=N—O—CH_2—CO—Arg_6 > Dau=N—O—CH_2—CO—Arg_8), while in the hydrazone linkage-group the presence of eight Arg residues provides the most efficiently trafficking variant (H—Glu(Arg_8)—NH—N=Dau > H—Glu(Arg_6)—NH—N=Dau).

In case of HepG2 cells, the uptake of the conjugates is dramatically lower (Table II, Figures 7 and 8). The measured average of mean fluorescence intensity values for HL-60 are in the range of 1868 and 4391, while for HepG2 cells data fall into a much lower range (250–579). It is important to add that similar tendency was identified by the comparison of the uptake of free Dau at $c = 20$ μM (fluorescence intensity values are 48621 for HL-60 and 10131 for HepG2 cells; Table II).

Interestingly, conjugates with oxime linkage proved to be taken up more efficiently. Dau=N—O—CH_2—CO—Arg_6 had the highest membrane translocation on HL-60 cells and Dau=N—O—CH_2—CO—Arg_8 by HepG2 cells at $c = 20$ μM (Figure 8).

DISCUSSION

Dau is widely used in cancer chemotherapy. Further development of its efficacy could cover the synthesis of analogues and also conjugates for tumor cell targeting. Dau can be conjugated to carrier molecules by various coupling techniques at four suitable sites.[11,22,25,26] The amino group of the daunosamine moiety, the 13-oxo group, the 4-methyl ether group, and the modified 14-methyl group of the anthracycline ring are used mostly for conjugation. The identity of the covalent link as well as the site of coupling could influence the chemical and also the biological properties of the conjugate. To establish correlation between structural and functional properties a new set of Dau-conjugates was prepared and comparative investigation was performed. A group of oligoarginine peptides was selected as carrier for conjugation. It has been reported that oligoarginines containing five or more Arg residues are considered as cell-penetrating peptides capable to translocate covalently attached cargo into the

cytosol.[33] The analysis of cell-penetrating properties are in the focus of intense research but the mechanism by which these compounds enter the cells is still not completely clear.[36]

We synthesized and characterized a group of novel Dau–oligoarginine conjugates containing different linkage (squaric amide, oxime, or hydrazone bond) and different number of Arg residues (6 or 8). We have produced for the first time modified oligoarginine peptides containing aminooxy or hydrazide group for conjugation. Via the amino group of Dau, squaric amide conjugates were prepared. Considering that oligoarginines are essentially insoluble in alcohol daunomycinyl-arginyl squaric acid diamide was synthesized and then this compound was coupled with oligoarginines in DMSO. Oxime and hydrazone conjugates were prepared from Dau and modified oligoarginine tayloring procedures described for peptide dendrimers.[54] The reactions proceeded smoothly, no side product formation was observed.

As reported earlier, Dau conjugates were mostly purified by RP-HPLC with eluent containing TFA.[8,27,55] These methods would have been satisfactory for conjugates with squaric amide and oxime linkage, but new approach had to be developed for the purification and chromatographic analysis of the highly acid-sensitive hydrazone conjugates. The stability studies of conjugates in buffers of different pH (pH = 2 and 5) and in distilled water suggest that squaric acid and oxime conjugates are stable under these circumstances over 2 weeks, while free Dau was released from the hydrazone conjugate even within a short period of time (half-life times for H—Glu(Arg_6)—NH—N=Dau are 25 min, 70 min, and 2 days at pH = 2, 5, 7, respectively). All Dau–oligoarginine conjugates were unstable after RP-HPLC purification, when TFA containing eluent was used. It is known that TFA could remain non-covalently bound to the basic guanidine group of arginine.[27] This could cause the split and liberation of daunosamino group of Dau even weeks after the purification. Therefore, a procedure to remove TFA by using gel chromatography and MCI gel was developed. In this way, the hydrazone conjugates were successfully purified and the stability of all conjugates in solid form were also improved. Furthermore, the new analytical RP-HPLC method was described, which could be suitable to monitor hydrazone or other acid-sensitive peptide conjugates.

Cytostatic effect and cellular uptake properties of Dau-conjugates were analyzed in vitro on HL-60 human leukemia and HepG2 human hepatoma cell lines. We found that the conjugates might have at least two properties which could influence their IC_{50} value. The first one is the coupling site on Dau involved for conjugation with oligoarginine : amino group or oxo group. According to the literature, the presence of the free amino group is essential for the effectiveness of Dau;[7] while the oxo-group could be modified without great loss of activity.[22]

The second factor is the stability of the chemical bond between Dau and the oligopeptide: oxime and squaric amide bonds are stable under conditions studied, while the hydrazone bond is an acid-sensitive linkage.[10] Conjugates with hydrazone bond in acidic circumstances would release free Dau more rapidly as compared with conjugates with stable (amide or oxime) bonds.

We found that Dau-peptide conjugates even with different type of covalent linkage were taken up more efficiently by HL-60 than by HepG2 cells. IC_{50} values of the compounds were mostly higher on HepG2 cells as compared with those obtained on HL-60 cells. This indicates that Dau-conjugates were more effective on HL-60 cells, than on HepG2 cells. This could be explained by the differences between these two cell lines.[56] Namely, for example HL-60 cells could be cultured in suspension, while HepG2 cells are adherent cells. Furthermore, these cells have different function, morphology, division, and more likely Dau-conjugates could be exposed also to different degradation mechanisms in HL-60 and HepG2 cells. Our findings are in harmony with those described by Nakase et al. on the influence of cell type of the mechanism of cellular uptake of oligoarginines.[36]

Taken together our findings indicate that the position and the type of chemical linkage between Dau and oligoarginine, as well as its acid-stability could have a marked influence on the uptake properties as well as on the intracellular events and routes (e.g., kinetics of degradation, trafficking) of the conjugates. Our data also suggest that the IC_{50} value of the compounds studied depends on the effectiveness of the cellular uptake, the coupling site applied for conjugation and the acid sensitivity of the linkage. Further studies are needed to investigate the type of the uptake mechanism[57] and also the degradation of the different peptide conjugates which could be an essential factor governing the IC_{50} value. Data presented in this communication could be considered also as a contribution to the rational design of improved antitumor agents.

Ms. Zsanett Miklán acknowledges the professional aid of Mihály Dernovics (Department of Applied Chemistry, Corvinus University Budapest, Hungary) during the development of HPLC method.

REFERENCES

1. Gaál, D.; Hudecz, F. Eur J Cancer 1998, 34, 155–161.
2. Baurain, R.; Masquelier, M.; Deprezde, C. D.; Trouet, A. Drugs Exp Clin Res 1983, 9, 303–311.
3. Nogusa, H.; Hamana, H.; Uchida, N.; Maekawa, R.; Yoshioka, T. Jpn J Cancer Res 2000, 91, 1333–1338.
4. Shiah, J. G.; Sun, Y. G.; Peterson, C. M.; Straight, R. C.; Kopecek, J. Clin Cancer Res 2000, 6, 1008–1015.

5. Kamada, H.; Tsutsumi, Y.; Yoshioka, Y.; Yamamoto, Y.; Kodaira, H.; Tsunoda, S.; Okamoto, T.; Mukai, Y.; Shibata, H.; Nakagawa, S.; Mayumi, T. Clin Cancer Res 2004, 10, 2545–2550.
6. Hurwitz, E. Biopolymers 1983, 22, 557–567.
7. Zunino, F.; Dimarco, A.; Velcich, A. Cancer Lett 1977, 3, 271–275.
8. Langer, M.; Kratz, F.; Rothen-Rutishauser, B.; Wunderli-Allenspach, H.; Beck-Sickinger, A. G. J Med Chem 2001, 44, 1341–1348.
9. Krauss, U.; Kratz, F.; Beck-Sickinger, A. G. J Mol Recognition 2003, 16, 280–287.
10. Rodrigues, P. C. A.; Roth, T.; Fiebig, H. H.; Unger, C.; Mulhaupt, R.; Kratz, F. Bioorg Med Chem 2006, 14, 4110–4117.
11. Masquelier, M.; Baurain, R.; Trouet, A. J Med Chem 1980, 23, 1166–1170.
12. Wong, B. K.; Defeo-Jones, D.; Jones, R. E.; Garsky, V. M.; Feng, D. M.; Oliff, A.; Chiba, M.; Ellis, J. D.; Lin, J. H. Drug Metab Dispos 2001, 29, 313–318.
13. Shen, W. C.; Ryser, H. J. P. Biochem Biophys Res Commun 1981, 102, 1048–1054.
14. Hudecz, F.; Clegg, J. A.; Kajtar, J.; Embleton, M. J.; Szekerke, M.; Baldwin, R. W. Bioconjug Chem 1992, 3, 49–57.
15. Rousselle, C.; Clair, P.; Lefauconnier, J. M.; Kaczorek, M.; Scherrmann, J. M.; Temsamani, J. Mol Pharmacol 2000, 57, 679–686.
16. Trouet, A.; Masquelier, M.; Baurain, R.; Deprezdecampeneere, D. Proc Natl Acad Sci USA 1982, 79, 626–629.
17. Arnold, L. J. Methods Enzymol 1985, 112, 270–285.
18. Taatjes, D. J.; Fenick, D. J.; Koch, T. H. Chem Res Toxicol 1999, 12, 588–596.
19. Kasiotis, K. M.; Magiatis, P.; Pratsinis, H.; Skaltsounis, A. L.; Abadji, V.; Charalambous, A.; Moutsatsou, P.; Haroutounian, S. A. Steroids 2001, 66, 785–791.
20. Tevyashova, A.; Sztaricskai, F.; Batta, G.; Herczegh, P.; Jeney, A. Bioorg Med Chem Lett 2004, 14, 4783–4789.
21. Shabat, D.; Rader, C.; List, B.; Lerner, R. A.; Barbas, C. F. Proc Natl Acad Sci USA 1999, 96, 6925–6930.
22. Yamamoto, K.; Acton, E.; Henry, D. J Med Chem 1972, 15, 872–875.
23. Griffiths, G. L.; Mattes, M. J.; Stein, R.; Govindan, S. V.; Horak, I. D.; Hansen, H. J.; Goldenberg, D. M. Clin Cancer Res 2003, 9, 6567–6571.
24. Ingallinella, P.; Di Marco, A.; Taliani, M.; Fattori, D.; Pessi, A. Bioorg Med Chem Lett 2001, 11, 1343–1346.
25. Capobianco, M. L.; De Champdore, M.; Arcamone, F.; Garbesi, A.; Gulanvarc'h, D.; Arimondo, P. B. Bioorg Med Chem 2005, 13, 3209–3218.
26. Horton, D.; Priebe, W.; Sznaidman, M. Carbohydr Res 1988, 184, 231–235.
27. Meyer-Losic, F.; Quinonero, J.; Dubois, V.; Alluis, B.; Dechambre, M.; Michel, M.; Cailler, F.; Fernandez, A. M.; Trouet, A.; Kearsey, J. J Med Chem 2006, 49, 6908–6916.
28. Banoczi, Z.; Peregi, B.; Orban, E.; Szabo, R.; Hudecz, F. Arkivoc 2008, 140–153.
29. Mezo, G.; Manea, M.; Szabo, I.; Vincze, B.; Kovacs, M. Curr Med Chem 2008, 15, 2366–2379.
30. Derossi, D.; Joliot, A. H.; Chassaing, G.; Prochiantz, A. J Biol Chem 1994, 269, 10444–10450.
31. Fawell, S.; Seery, J.; Daikh, Y.; Moore, C.; Chen, L. L.; Pepinsky, B.; Barsoum, J. Proc Natl Acad Sci USA 1994, 91, 664–668.
32. Mitchell, D. J.; Kim, D. T.; Steinman, L.; Fathman, C. G.; Rothbard, J. B. J Pept Res 2000, 56, 318–325.
33. Futaki, S.; Suzuki, T.; Ohashi, W.; Yagami, T.; Tanaka, S.; Ueda, K.; Sugiura, Y. J Biol Chem 2001, 276, 5836–5840.
34. Wender, P. A.; Mitchell, D. J.; Pattabiraman, K.; Pelkey, E. T.; Steinman, L.; Rothbard, J. B. Proc Natl Acad Sci USA 2000, 97, 13003–13008.
35. Patel, L. N.; Zaro, J. L.; Shen, W. C. Pharm Res 2007, 24, 1977–1992.
36. Nakase, I.; Takeuchi, T.; Tanaka, G.; Futaki, S. Adv Drug Deliv Rev 2008, 60, 598–607.
37. Hudecz, F.; Banoczi, Z.; Csik, G. Med Res Rev 2005, 25, 679–736.
38. Foged, C.; Nielsen, H. M. Expert Opin Drug Deliv 2008, 5, 105–117.
39. Kirschberg, T. A.; VanDeusen, C. L.; Rothbard, J. B.; Yang, M.; Wender, P. A. Org Lett 2003, 5, 3459–3462.
40. Viht, K.; Padari, K.; Raidaru, G.; Subbi, J.; Tatummiste, I.; Pooga, M.; Uri, A. Bioorg Med Chem Lett 2003, 13, 3035–3039.
41. Miklan, Z.; Szabo, R.; Zsoldos-Mady, V.; Remenyi, J.; Banoczi, Z.; Hudecz, F. Biopolymers 2007, 88, 108–114.
42. Rothbard, J. B.; Garlington, S.; Lin, Q.; Kirschberg, T.; Kreider, E.; McGrane, P. L.; Wender, P. A.; Khavari, P. A. Nat Med 2000, 6, 1253–1257.
43. Yang, L. L.; Mashima, T.; Sato, S.; Mochizuki, M.; Sakamoto, H.; Yamori, T.; Oh-hara, T.; Tsuruo, T. Cancer Res 2003, 63, 831–837.
44. Morishita, M.; Kamei, N.; Ehara, J.; Isowa, K.; Takayama, K. J Control Release 2007, 118, 177–184.
45. Kaiser, E.; Colescott, R.; Bossinger, C.; Cook, P. Anal Biochem 1970, 34, 595–598.
46. Arndt, F. In Diazomethane.Organic Synthesis, Collective, Vol. 2; Blatt, A. H., Ed.; John Wiley & Sons: New York, 1943; pp 165–167.
47. Sztaricskai, F.; Sum, A.; Roth, E.; Pelyvas, I. F.; Sandor, S.; Batta, G.; Herczegh, P.; Remenyi, J.; Miklan, Z.; Hudecz, F. J Antibiotics 2005, 58, 704–714.
48. Collins, S. J.; Gallo, R. C.; Gallagher, R. E. Nature 1977, 270, 347–349.
49. Slater, T. F.; Sawyer, B.; Strauli, U. Biochim Biophys Acta 1963, 77, 383–393.
50. Miklán, Z.; Reményi, J.; Schlosser, G.; Hudecz, F. XVIX Meeting of the European Association for Cancer Research, Budapest, 2006, Abstract 101, p 84.
51. Andreoni, A.; Colasanti, A.; Roberti, G. J Photochem Photobiol B 1992, 14, 319–328.
52. Fujiwara, K.; Takatsu, H.; Tsukamoto, K. J Histochem Cytochem 2005, 53, 467–474.
53. Reményi, J.; Hegedus, T.; Kovács, P.; Csík, G.; Sarkadi, B.; Hudecz, F. FEBS J 2005, 272(S1), 528.
54. Shao, J.; Tam, J. P. J Am Chem Soc 1995, 117, 3893–3899.
55. Burkhart, D. J.; Kalet, B. T.; Coleman, M. P.; Post, G. C.; Koch, T. H. Mol Cancer Ther 2004, 3, 1593–1604.
56. Jin, W. R.; Shi, Q.; Hong, C. T.; Cheng, Y. Y.; Ma, Z. J.; Qu, H. B. Phytomedicine 2008, 15, 768–774.
57. Futaki, S. Adv Drug Deliv Rev 2005, 57, 547–558.