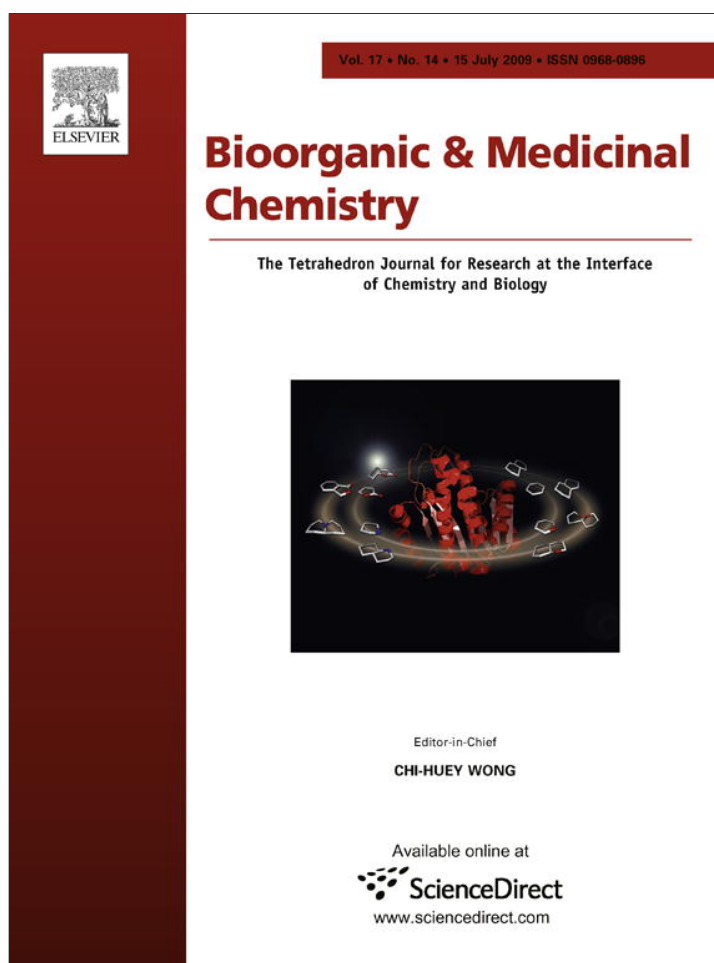


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Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Efficient synthesis of the first betulonic acid–acetylene hybrids and their hepatoprotective and anti-inflammatory activity

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ARTICLE INFO

Article history:

Received 23 June 2008

Revised 18 May 2009

Accepted 22 May 2009

Available online 29 May 2009

Keywords:

Cross-coupling

Arylacetylenes

Betulonic acid

Triterpenoids

Hepatoprotection

Anti-inflammatory activity

ABSTRACT

The Sonogashira reaction can be applied for the preparation of acetylenic derivatives of betulonic acid where the triterpenoid moiety can serve as either the halo- or the acetylenic component. This reaction opened access to the first derivatives of betulonic acid containing either the arylethynyl (C≡C-Ar(Het)) or the ethynyl (C≡CH) moieties. From the fundamental perspective, this work illustrates the possibility of selective Pd-catalyzed cross-coupling at terminal acetylenes in the presence of a terminal alkene. Hepatoprotective and anti-inflammatory properties of selected acetylenic derivatives of betulonic acid were investigated using the CCl₄-induced hepatitis and carrageenan-induced edema models, respectively.

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1. Introduction

Plant-derived pentacyclic triterpenoids of lupane and oleanane families provide a convenient structural platform for synthetic studies aimed at the discovery of new biologically active compounds. A number of semisynthetic derivatives of these ubiquitous molecules, sometimes obtained through relatively simple transformations, possess high medical efficiency. In particular, research published in the last decade has emphasized such pharmacological properties of oleanane and lupane derivatives as antiviral (HIV-1), anticancer and immunomodulating activity.¹ In the process of further development of our research program dedicated to the investigation of plant metabolites produced by trees and herbs of Siberia,² we describe efficient synthesis and selected medicinal properties of betulonic acid derivatives containing acetylenic structural units at the C28 position. Introduction of substituents at this position,³ in particular through the formation of amides,⁴ provided highly promising results in the search of antitumor agents in the past.

Despite high biological activity of many natural acetylenic metabolites,^{5,6} acetylenic derivatives of betulonic acid remained previously unknown. Moreover, only a few tertiary acetylenic alco-

hols of the triterpenoid family (derived from 3-keto-glycyrrhetic acid)⁷ has been described in the literature. We were particularly intrigued by the possibility of combining the recent observations of significant synergistic effect of irradiation on the effect of betulonic acid derivatives on survival and growth inhibition of several melanoma cell lines increases^{8a} with the ability of aryl acetylenes to induce efficient double-stranded DNA photocleavage.^{8b,c} Recently, the alkyne moiety became one of the most commonly used substituents in the design of new medicinal agents due to the ability of alkynes to undergo facile cycloaddition reactions useful for the modular construction of polyfunctional architectures and for labeling biomolecules *in vitro*.⁹

We envisioned two approaches to such acetylenic derivatives. Both of them take advantage of the acyl chloride functionality for the attachment of substituted anilines. In the first approach, the reaction with amino-substituted phenylacetylene allows preparation of the key precursor **1**—derivative of betulonic acid which possesses a terminal ethynyl moiety useful for a variety of further transformations. The second approach is based on the preparation of an iodo derivative through the condensation with *p*-iodoaniline and subsequent Sonogashira coupling of the resulting aryl iodide with terminal alkynes.

In both of these cases, we were interested in determining conditions where the alkene functionality of the isopropylidene moiety characteristic for the lupane family does not interfere with the Sonogashira cross-coupling. Success of such couplings has

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not been a priori obvious because the vinyl group is known to react with aryl halides with the formation of disubstituted olefins (the Heck reaction). The two reactions proceed under analogous conditions and are catalyzed by the same Pd complexes (such as $\text{PdCl}_2(\text{PPh}_3)_2$ and $\text{Pd}(\text{OAc})_2$).¹⁰ As a consequence, both the Heck and the Sonogashira reactions can proceed in parallel for the first of the above approaches because the substrate can either alkenylate itself (Heck) or be alkynylated by a terminal alkyne (Sonogashira). A similar problem could potentially complicate the cross-coupling reactions of betulonic acid derivatives which contain both the vinyl and the ethynyl residues (approach 1), both of which can participate in the cross-coupling reactions.

Considering the above, we concentrated our efforts on the first approach because presence of the terminal alkyne moiety in the key triterpene alkyne synthetic intermediate **1** (prepared in 55% yield through the interaction of the acyl chloride **2** with 4-aminophenylacetylene in the presence of triethylamine, *Scheme 1*) opens a number of possibilities for further synthetic modifications which are not limited to the cross-coupling reactions.

In particular, interaction with piperidine and morpholine and paraform under the Mannich reaction conditions (*Scheme 2*) yielded 69% and 75% of the propargyl amines **3a**, **3b**. This compound is potentially interesting considering the data regarding the activity of 3-arylprop-2-ynyl amines as squalene epoxidase (squalene monooxygenase) inhibitors in mammals.¹¹

The Cadiot–Chodkiewicz cross-coupling is one of important synthetic methods traditionally used for the preparation of diacetylene derivatives including α -diacetylenic alcohols, which were found to possess high anticancer activity.⁵ A number of naturally occurring conjugated butadiynyl carbinols have been prepared by this method.¹² Gratifyingly, the reaction of **1** with dimethyl-3-bromopropynol in methanol in the presence of CuCl , Et_3N and $\text{NH}_2\text{OH}\cdot\text{HCl}$ proceeded without complications and afforded α -diacetylenic alcohol **4** in 73% yield.

Considering the polyfunctional character of betulonic acid, it was important to test the possibility of cross-coupling of acetylene **1** with haloarenes. If successful, this will open synthetic access to a wide variety of betulonic acid derivatives with ethynylaryl and ethynylhetaryl moieties.

As mentioned above, a possible complication could arise from the presence of the vinyl and the ethynyl residues in the reacting molecule. From the literature, it is known that the Heck reaction can involve not only aryl-, but also alkyl- and cycloalkenes.¹³ In order to test the possibility of achieving selective alkynylation, we have chosen 2-bromopyridine as the halogen component. This choice was based on the observation of R. F. Heck that halogen derivatives are more reactive towards alkynes than towards alkenes.¹⁴

Under standard Sonogashira conditions ($\text{PdCl}_2(\text{PPh}_3)_2$, CuI , Et_3N), interaction of ethynyl derivative **1** with 2-bromopyridine (55 °C, 18 h) provides only the alkynylation product—the disubstituted acetylene **5** in 62%. Such selectivity is likely to result from higher reactivity of halides towards Sonogashira reactions than in the Heck reaction. Although these findings are consistent with

the earlier literature observations, the relative cross-coupling reactivity of an alkene and an alkyne moiety from the same molecule, to the best of our knowledge, has not been studied before.

These results stimulated us to investigate whether the ‘inverse’ version of the Sonogashira reaction with the betulonic acid derivative as a halogen component would also be possible. Again, the success of this reaction has not been a priori guaranteed because the iodobetulonic acid can also act as the olefin component and, thus, one can expect the mixture of the Heck alkenylation and Sonogashira alkynylation.

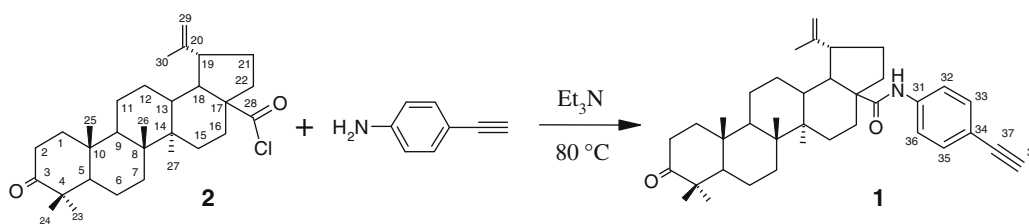
The requisite iodoamide **6** has been synthesized in the 50% yield through the interaction of 4-iodoaniline with acid chloride **2** in the presence of triethylamine (*Scheme 3*). A very interesting feature of the subsequent coupling step is the direct introduction of terminal 1,3-butadiynyl moiety in Sonogashira reaction. This is not a trivial task because of the instability of molecules with the terminal diacetylenic moiety.¹⁵

1-Hydroxy-2-methylhexa-3,5-diyne, which serves as the alkyne component, has been previously synthesized via oxidative coupling of dimethylethynyl carbinol to diacetylenic glycole followed by its selective fragmentation through the retro Favorsky reaction in the combined yield of 56%.¹⁶ We utilized a significantly improved procedure developed by one of us¹⁷ where the key modification for increasing the reaction efficiency involved heating of the diacetylenic glycole with K_2CO_3 in *s*-pentaphenyl ether, a high-boiling non-volatile solvent which minimizes thermal decomposition. In this procedure, the acetylene is distilled off under the aspirator vacuum without any thermal decomposition whereas *m*-pentaphenyl is left in the distillation residue. The yield of twice distilled diacetylene is 92%.

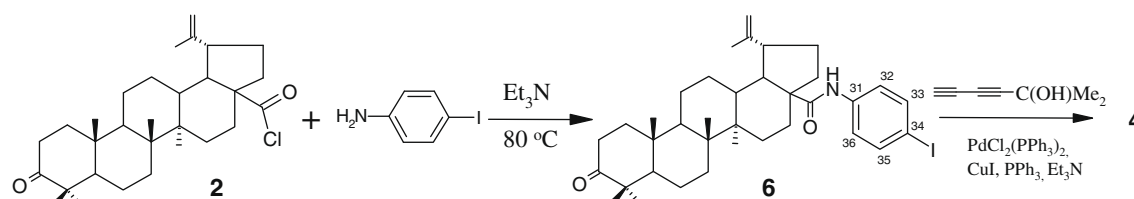
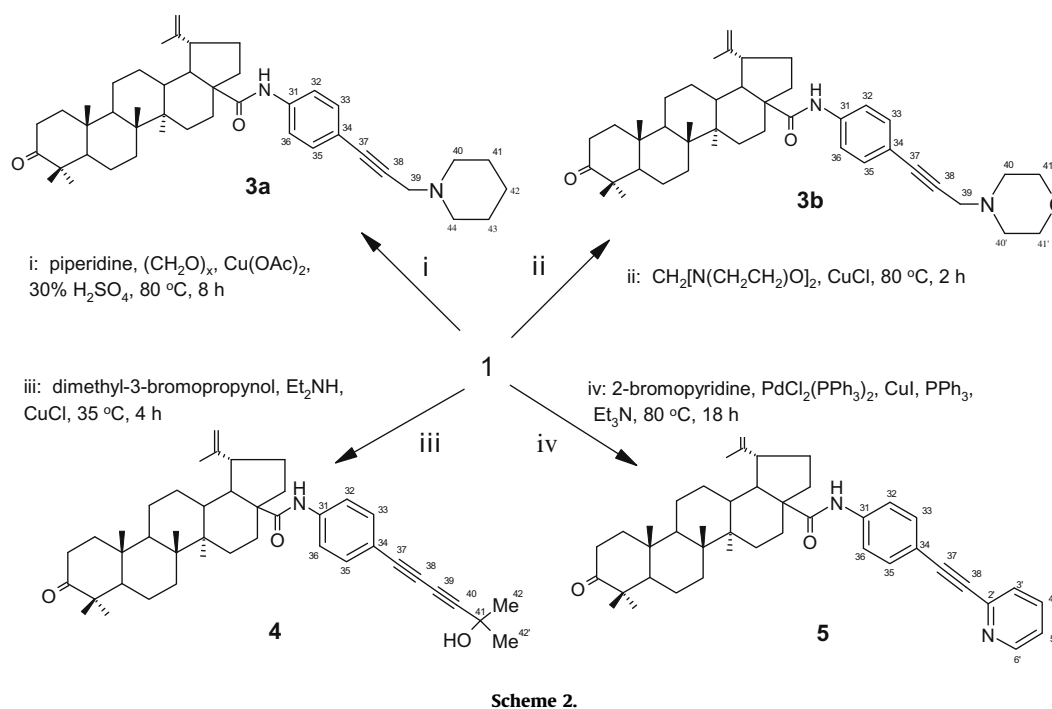
Interaction of iodobetulonic acid **6** with the diacetylenic alcohol proceeded under the cross-coupling conditions analogous to those utilized for alkyne **1** and α -bromopyridine ($\text{PdCl}_2(\text{PPh}_3)_2$, CuI , Et_3N , 55 °C) and provided the target 1,3-butadiyne **4** in the 57% yield.

The above transformation also presents an alternative synthesis of diacetylenyl betulonic acid obtained from the Cadiot–Chodkiewicz reaction (see *Scheme 2*). Comparison of these two approaches suggests that both of them can be used for the efficient preparation of butadiynyl lupane derivatives.

Structure of the prepared acetylenic derivatives of betulonic acid has been established from the analysis of spectral data (IR, ^1H and ^{13}C NMR). 2D-spectra (COSY) and ^1H – ^{13}C correlations (COSY, COLOS) were used for the full assignment of the ^{13}C NMR signals of compounds **4** and **5**. In particular, the unambiguous assignment of C-39 of the diyne moiety and C-41 (singlets at 65.27 and 66.65 ppm, respectively) for compound **4** from the cross-peaks between carbon C-41 and hydrogens of the methyl groups (C^{42}H_3 and C^{42}H_3 at 1.54 ppm) in the ^1H – ^{13}C COLOC spectrum. Correlation with the above methyl protons is also observed for the C-40 carbon (δ 86.24 ppm), unlike the C-37 which displays cross peak with H-33, 35 (7.43 ppm). Assignment of carbons C-37, 38 (s, 88.79 and 88.75) of the alkyne moiety in compound **5** is confirmed by the presence of a cross-peak between aromatic hydrogens at C-33, 35 (7.50 ppm) and carbon C-37.



Scheme 1.



2. Biological activity: hepatoprotective and anti-inflammatory properties of acetylenic derivatives of betulonic acid

The hepatoprotective and anti-inflammatory properties of compounds described in the previous section were investigated using the CCl_4 -induced hepatitis and carrageenan-induced edema models, respectively. These models were described in detail in the experimental part.

The results of biochemical analysis of blood serum of animals with toxic hepatitis (Table 1) show that all compounds decrease the alkaline phosphatase activity, which confirms their anticholestatic properties. Compound **6** displayed the most apparent effect comparable with that of dihydroquercetin. Compound **3a** has a moderate effect whereas compound **3b** shows weak anticholestatic properties. It is found that two of the studied compounds decrease

the ALT and AST activity in blood as a result of anticytolytic effect. Betulonic derivatives **6** and **3a** lower the transaminase activity 1.8- and 1.6-fold whereas dihydroquercetin causes only a 1.4-fold decrease in the activity. Compound **3b** is inferior to dihydroquercetin in this regard. None of the compounds display significant antioxidant activity under the above experimental conditions. Nevertheless, derivative **3a** lowered the TBARS concentration in the blood 1.5 times group whereas dihydroquercetin decreased this parameter only by factor of 1.2. These results suggest that further studies of analogues of compound **3a** in this direction may be warranted.

In the carrageenan-induced paw edema model, only compound **3a** displayed a statistically significant effect by lowering the edema index 1.4 times relatively to the control group (Table 2). Compound **3b** showed only a tendency to the anti-inflammatory effect whereas derivative **6** did not exhibit an appreciable effect. It is

Table 1
Effect of betulonic derivatives on biochemical parameters in the blood serum of mice with CCl_4 -hepatitis

| Agent | Dose (mg/kg) | ALP, $\mu\text{cat/L}$ | ALT, $\mu\text{cat/L}$ | AST, $\mu\text{cat/L}$ | TBARS, $\mu\text{mol/l}$ |
|------------------|--------------|------------------------|------------------------|------------------------|--------------------------|
| Control | — | 18.27 \pm 1.42 | 5.04 \pm 0.72 | 5.23 \pm 0.76 | 1.63 \pm 0.24 |
| 3b | 50 | 14.28 \pm 1.50*** | 3.48 \pm 0.45* | 5.25 \pm 0.66 | 3.21 \pm 0.62* |
| 6 | 20 | 10.27 \pm 0.64*** | 2.75 \pm 0.42* | 3.09 \pm 0.53* | 2.03 \pm 0.31 |
| 3a | 50 | 12.80 \pm 1.53** | 3.08 \pm 0.34* | 3.25 \pm 0.38* | 1.06 \pm 0.14 |
| Dihydroquercetin | 100 | 11.31 \pm 1.13** | 3.63 \pm 0.59 | 3.88 \pm 0.70 | 1.42 \pm 0.29 |

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ —differences with control are significant.

Table 2
Effect of betulonic derivatives on the index of paw edema indexes in carrageenan-induced edema model

| Control | 3b | 6 | 3a | Indomethacin |
|------------|-------------------------|---------------------------|--------------------------|---------------------------|
| 75.5 ± 3.1 | 65.8 ± 3.6 [#] | 77.2 ± 3.4 ^{###} | 56.1 ± 4.5 ^{**} | 51.9 ± 3.9 ^{***} |

^{**} $p < 0.01$, ^{***} $p < 0.001$ —differences with control are significant.

[#] $p < 0.05$, ^{###} $p < 0.001$ —differences with indomethacin are significant.

noteworthy that compound **3a** has anti-inflammatory activity which is comparable to that of indomethacin.

In conclusion, we have shown that the Sonogashira reaction is applicable for the preparation of acetylenic derivatives of betulonic acid and that the triterpenoid moiety can serve as either the halo- or the acetylenic component. This reaction has opened access to first derivatives of betulonic acid containing either the arylethynyl (C≡C-Ar(Het)) or the ethynyl (C≡CH) moieties. These highly reactive triterpenoid derivatives open broad possibilities for the preparation of new families of promising compounds for the development of biologically active molecules for medicinal applications.¹⁸

A fundamentally important conclusion from the present work is the possibility of selective Pd-catalyzed cross-coupling at the terminal acetylenic carbon of the ethynyl derivative of betulonic acid in the presence of a terminal alkene substituent which is also reactive towards haloarenes and heteroarenes under these conditions. This is a useful finding because efficient chemical processes for practical applications should involve the minimum number of protecting group manipulations.

Similar selectivity was found during the reaction of the iodo derivative with diacetylenic alcohol—only alkynylation product of the Sonogashira reaction is isolated, no alkenylation product of the Heck reaction is observed even though the starting material has both the halo- and the olefin components for the Heck cross-coupling. Hepatoprotective and anti-inflammatory properties of selected acetylenic derivatives of betulonic acid were investigated using the CCl₄-induced hepatitis and carrageenan-induced edema models. These studies established that compound **3a** has significant hepatoprotective, anti-inflammatory effects and a potential antioxidant effect. Compound **6** displayed significant hepatoprotective properties, whereas compound **3b** showed no significant hepatoprotective and anti-inflammatory activities. These observations indicate that biological properties of this family of compounds are sensitive to the nature of substituents and, thus, justify synthetic studies aimed at the preparation of this promising class of medicinally interesting compounds.

3. Experimental

Melting points were determined with a Kofler apparatus. Column chromatography was performed on Al₂O₃ ('Aldrich') and the Silufol UV-254 plates were used for TLC analysis. The IR-spectra were recorded in KBr pellets on a 'Bruker IFS 66' instrument. PdCl₂(PPh₃)₂, methyl-3-butyn-2-ol, Et₃N, CuCl ('Aldrich') were commercially available reactants. All the organic solvents were of analytical quality. Mass spectra (HRMS) were measured on a 'Finnigan MAT', model 8200, 70 eV. Combustion analysis was performed with CHN-analyzer (Model 1106, 'Carlo Erba', Italy).

NMR spectra were recorded on a 'Bruker Avance 300' spectrometer at (300.13 (¹H) and 75.47 MHz (¹³C)) and on a 'Bruker AM-400' 400.13 (¹H) and 100.61 MHz (¹³C) at 25 °C. Structural assignments in the NMR spectra were carried out by means of proton-proton and carbon-proton correlation spectroscopy (COSY, COLOC) using Bruker DRX-500 (500.13 (¹H) и 125.76 MHz (¹³C)) for compounds **4** and **5**, taking into account the available ¹³C assignments for betu-

lonic acid.¹⁹ Chemical shifts were given in (δ in ppm) relative to the residual signals of CHCl₃ (δ_{H} 7.24 ppm and δ_{C} 76.90 ppm). Coupling constants (J in Hz) were accurate to ± 0.2 Hz. The multiplicity of signals in the ¹³C NMR spectra was determined using J-modulation (JMOD). Due to the complexity of signals in the ¹H NMR spectra for the betulonic acid derivatives, only the characteristic signals are assigned. Most protons of the triterpenoid skeleton resonate between 0.8 and 2.7 ppm. Assignment of the alkyne carbons in the ¹³C NMR was carried out through comparison with the respective chemical shifts of alkynes described earlier.²⁰

3.1. N-(3-Oxolup-20(29)en-28-oyl)-4-ethynylaniline **1**

1.6 g (3.4 mmol) of the chloroanhydride **2**, 0.39 g (3.4 mmol) of *p*-aminophenylacetylene and 3 mL of triethylamine were added to a flask with 15 mL of dry benzene under the argon atmosphere. The flask was fitted with a reflux condenser and kept at 70–75 °C for 18 h. The Et₃N·HCl precipitate was filtered off and washed with benzene (3 × 10 mL). Solvent was removed in vacuo. The residue was dissolved in benzene and washed with diluted HCl (1:4), dried over anhydrous Na₂SO₄ and concentrated. The concentrated solution was filtered through alumina (2 × 2.5 cm) and washed with benzene. Solvent was removed in vacuo to afford 1.0 g (55%) of compound **1**, mp 159–160 °C (benzene).

HRMS, found: m/z 553.3918 [M]⁺. C₃₈H₅₁NO₂. Calcd: $M = 553.3919$. ¹H NMR (CDCl₃, 300.13 MHz) δ 0.90 (3H, s, Me-25), 0.95 (3H, s, Me-24), 0.98 (6H, s, Me-26, 27), 1.03 (3H, s, Me-23), 1.67 (3H, s, Me-30), 3.14 (1H, dt, 19, $J_1 = 4$ Hz, $J_2 = 11$ Hz), 4.59 (1H, s, 29), 4.73 (1H, s, 29), 3.01 (1H, s, 38), 7.39 (4H, m, 32, 36, 33, 35); ¹³C NMR δ 14.37 (C-27), 15.77 (C-26), 15.82 (C-25), 19.32 (C-30), 19.43 (C-6), 20.82 (C-24), 21.27 (C-11), 25.45 (C-12), 26.38 (C-23), 29.41 (C-21), 30.60 (C-15), 33.53 (C-16), 33.63 (C-7), 33.93 (C-2), 36.76 (C-22), 37.49 (C-13), 37.85 (C-10), 39.46 (C-1), 40.55 (C-8), 42.43 (C-14), 46.27 (C-19), 47.13 (C-4), 49.86 (C-18), 50.07 (C-9), 54.92 (C-5), 56.45 (C-17), 62.41 (C-37), 86.76 (C-38), 109.42 (C-29), 116.29 (C-34), 121.75 (C-32, 36), 137.66 (C-33, 35), 137.89 (C-31), 150.30 (C-20), 174.31 (C-28), 217.73 (C-3); IR, cm⁻¹, ν : 1696 (C=O), 2108 (C≡C), 3387 (C≡C-H); Anal. Calcd for C₃₈H₅₁NO₂: C, 82.32; H, 8.72; N, 2.38. Found: C, 82.41; H, 9.28; N, 2.53.

3.2. N-(3-Oxolup-20(29)en-28-oyl)-4-(N-piperidinepropargyl)-aniline **3a**

A mixture of 25 mg (0.85 mmol) of paraform and 72 mg (0.85 mmol) of piperidine in to 5 mL of dioxane was stirred under argon. After the reaction was kept at 55 °C for 30 min, 8 mg (0.047 mmol) of Cu(OAc)₂, 0.2 mL of 30% H₂SO₄ and 221 mg (0.4 mmol) of compound **1** were added and stirred at 80 °C for 8 h. After the reaction was complete, the reaction mixture was transferred to a separatory funnel and washed with aqueous ammonia to remove the copper salts. The organic layer was separated, dried over Na₂SO₄ and filtered. The solvent was removed in vacuo. Hexane was added and the resulting precipitate was filtered off to afford 180 mg (69%) of the Mannich base, mp 137–139 °C (benzene).

HRMS, Found: m/z 650.4821 [M]⁺. C₄₄H₆₂N₂O₂. Calcd: $M = 650.4806$. ¹H NMR (CDCl₃, 400.13 MHz) δ 0.88 (3H, s, Me-25), 0.91 (3H, s, Me-24), 0.96 (6H, s, Me-26, 27), 1.04 (3H, s, Me-23), 1.66 (3H, s, Me-30), 2.55 (4H, m, 40, 44), 3.13 (1H, dt, 19, $J_1 = 4$ Hz, $J_2 = 11$ Hz), 3.44 (2H, s, CH₂-39), 4.60 (1H, s, 29), 4.75 (1H, s, 29), 7.40 (4H, m, 32, 36, 33, 35); ¹³C NMR δ 14.42 (C-27), 15.78 (C-26), 15.83 (C-25), 19.36 (C-30), 19.47 (C-6), 20.89 (C-24), 21.30 (C-11), 23.75 (C-41, 43), 25.46 (C-12), 25.71 (C-42), 26.41 (C-23), 29.44 (C-21), 30.61 (C-15), 33.54 (C-16), 33.69 (C-7), 34.02 (C-2), 36.79 (C-22), 37.48 (C-13), 37.95 (C-10), 39.50

(C-1), 40.56 (C-8), 42.46 (C-14), 46.29 (C-19), 47.21 (C-4), 48.31 (C-39), 49.88 (C-9), 50.06 (C-18), 54.91 (C-5), 53.30 (C-40, 44), 56.45 (C-17), 84.24 (C-38), 84.59 (C-37), 109.47 (C-29), 118.49 (C-34), 119.39 (C-32, 36), 132.27 (C-33, 35), 137.89 (C-31), 150.42 (C-20), 174.28 (C-28), 218.01 (C-3). IR, cm^{-1} , ν : 1710 (C=O); 2227 (C≡C). Anal. Calcd for $\text{C}_{44}\text{H}_{62}\text{N}_2\text{O}_2$: C, 81.18; H, 9.60; N, 4.30. Found: C, 80.85; H, 9.91; N, 4.15.

3.3. N-(3-Oxolup-20(29)en-28-oyl)-4-(N-morpholinopropargyl)-1)aniline 3b

5.22 g (60 mmol) of morpholine was added to 1.8 g (60 mmol) of paraform in 15 mL of dioxane at room temperature. The mixture was heated to 70 °C with reflux condenser for 2–2.5 h until full dissolution of paraform. The reaction mixture was then filtered. Evaporation of solvent in vacuo provided 5 g (89.6%) of di(N-morpholino)methane, bp 97–109°/2 mm, n_D^{20} 1.4792 (lit. bp 87–92°/1 mm, n_D^{20} 1.4790).²¹

To the mixture of 260 mg (0.47 mmol) of acetylene **1**, 93 mg (0.5 mmol) of di(N-morpholino)methane in 10 mL of dioxane and 13 mg (0.13 mmol) of CuCl were added under argon atmosphere. The reaction mixture was kept at 80 °C for 2 h, diluted with 15 mL of dichloromethane and then washed with aqueous ammonia. The organic phase was dried over Na_2SO_4 , filtered and concentrated in vacuo. The product was precipitated with hexane to afford 230 mg (74.9%) of compound **3b**, mp 151–152.5 °C (benzene). HRMS, Found: m/z 652.4578 [M]⁺. $\text{C}_{43}\text{H}_{60}\text{N}_2\text{O}_3$. Calcd: $M = 652.4599$. ¹H NMR (CDCl_3 , 400.13 MHz) δ 0.90 (3H, s, Me-25), 0.94 (3H, s, Me-24), 0.98 (6H, s, Me-26, 27), 1.03 (3H, s, Me-23), 1.67 (3H, s, Me-30), 2.61 (4H, m, 40, 40'), 3.13 (1H, dt, 19, $J_1 = 4$ Hz, $J_2 = 11$ Hz), 3.44 (2H, s, CH₂-39), 3.75 (4H, m, 41, 41'), 4.59 (1H, s, 29), 4.73 (1H, s, 29), 7.37 (4H, m, 32, 36, 33, 35); ¹³C NMR δ 14.43 (C-27), 15.79 (C-26), 15.83 (C-25), 19.36 (C-30), 19.47 (C-6), 20.89 (C-24), 21.31 (C-11), 25.47 (C-12), 26.41 (C-23), 29.45 (C-21), 30.61 (C-15), 33.54 (C-16), 33.69 (C-7), 34.02 (C-2), 36.79 (C-22), 37.48 (C-13), 37.95 (C-10), 39.51 (C-1), 40.56 (C-8), 42.46 (C-14), 46.29 (C-19), 47.22 (C-4), 47.96 (C-39), 49.88 (C-9), 50.06 (C-18), 54.91 (C-5), 52.31 (C-40, 40'), 56.47 (C-17), 66.74 (41, 41'), 83.33 (C-38), 85.12 (C-37), 109.49 (C-29), 118.14 (C-34), 119.42 (C-32, 36), 132.29 (C-33, 35), 138.08 (C-31), 150.41 (C-20), 174.33 (C-28), 218.07 (C-3). IR, cm^{-1} , ν : 1703 (C=O); 2225 (C≡C).

3.4. N-(3-Oxolup-20(29)en-28-oyl)-4-(5-hydroxy-5-methylhexa-1,3-diyanyl)aniline 4

188 mg (0.339 mmol) of compound **1**, 0.2 mL of diethylamine, 3.4 mg (0.05 mmol) of hydroxylamine hydrochloride and 1 mg (0.01 mmol) of CuCl were added under argon to 2 mL of methanol upon stirring. After formation of the bright yellow precipitate of copper acetylide, 62 mg (0.38 mmol) of 1-bromo-3-methylbut-1-yn-3-ol was added. The reaction mixture was kept at 30–35 °C for 4 h, diluted with 20 mL of toluene and washed with aqueous ammonia. The organic phase was dried over Na_2SO_4 , filtered and concentrated in vacuo. The product was precipitated with hexane to afford 156 mg (73%) of compound **4**, mp 183–184 °C (benzene). ¹H NMR (CDCl_3 , 300.13 MHz) δ 0.89 (3H, s, Me-25), 0.94 (6H, s, Me-26, 27), 0.98 (3H, s, Me-24), 1.03 (3H, s, Me-23), 1.66 (3H, s, Me-30), 1.54 (6H, s, 42, 43), 3.12 (1H, dt, 19, $J_1 = 4$ Hz, $J_2 = 11$ Hz), 4.58 (1H, s, 29), 4.72 (1H, s, 29), 7.42 (4H, m, 32, 33, 35, 36); ¹³C NMR δ 14.10 (C-27), 15.48 (C-26), 15.52 (C-25), 19.04 (C-30), 19.15 (C-6), 20.58 (C-24), 20.99 (C-11), 25.13 (C-12), 26.10 (C-23), 29.12 (C-21), 30.26 (C-15), 30.68 (C-42, 42'), 33.21 (C-16), 33.32 (C-7), 33.71 (C-2), 36.47 (C-22), 37.15 (C-13), 37.59 (C-10), 39.19 (C-1), 40.24 (C-8), 42.14 (C-14), 45.95 (C-19), 46.91 (C-4), 49.55 (C-18), 49.71 (C-9),

54.59 (C-5), 56.23 (C-17), 65.27 (C-39), 66.65 (C-41), 72.30 (C-38), 78.20 (C-37), 86.24 (C-40), 109.22 (C-29), 116.16 (C-34), 119.14 (C-32, 36), 132.92 (C-33, 35), 138.73 (C-31), 150.03 (C-20), 174.10 (C-28), 217.88 (C-3); IR, cm^{-1} , ν : 1692 (C=O), 2145 and 2232 (C≡C). Anal. Calcd for $\text{C}_{43}\text{H}_{57}\text{NO}_3$: C, 81.17; H, 10.10; N, 2.01. Found: C, 81.21; H, 9.03; N, 2.20.

3.5. N-(3-Oxolup-20(29)en-28-oyl)-4-(2-ethynylpyridyl)aniline 5

Upon stirring, 78 mg (0.49 mmol) of α -bromopyridine, 7 mg (0.04 mmol) of CuI, 7 mg (0.01 mmol) of $\text{PdCl}_2(\text{PPh}_3)_2$, 4 mg (0.02 mmol) of PPh_3 , 300 mg (0.542 mmol) of acetylene **1** and 3 mL of triethylamine were added under argon to 10 mL of toluene. The mixture was heated to 55 °C with reflux condenser for 18 h. The $\text{Et}_3\text{N}\cdot\text{HBr}$ precipitate was filtered off and washed with toluene (3 \times 10 mL). Organic fractions were combined and solvent was evaporated in vacuo. The product was precipitated upon trituration with hexane and filtered to afford 193 mg (62%) of compound **5**, mp 181–182 °C (benzene). ¹H NMR (CDCl_3 , 300.13 MHz) δ 0.89 (3H, s, Me-25), 0.94 (3H, s, Me-24), 0.97 (6H, s, Me-26, 27), 1.06 (3H, s, Me-23), 1.66 (3H, s, Me-30), 3.13 (1H, dt, 19, $J_1 = 4$ Hz, $J_2 = 11$ Hz), 4.62 (1H, s, 29), 4.76 (1H, s, 29), 7.50 (4H, m, 32, 33, 35, 36), 7.46 (1H, d, 3', $J = 3$ Hz), 7.64 (1H, t, 4', $J_1 = 1.7$ Hz, $J_2 = 7.7$ Hz), 7.20 (1H, m, 5'), 8.58 (1H, d, 6', $J = 4.9$ Hz); ¹³C NMR δ 14.10 (C-27), 15.47 (C-26), 15.55 (C-25), 19.05 (C-30), 19.15 (C-6), 20.58 (C-24), 21.00 (C-11), 25.15 (C-12), 26.10 (C-23), 29.14 (C-21), 30.29 (C-15), 33.22 (C-16), 33.31 (C-7), 33.71 (C-2), 36.47 (C-22), 37.15 (C-13), 37.61 (C-10), 39.19 (C-1), 40.25 (C-8), 42.14 (C-14), 45.97 (C-19), 46.90 (C-4), 49.57 (C-18), 49.75 (C-9), 54.60 (C-5), 56.20 (C-17), 87.75 (C-38), 88.79 (C-37), 109.17 (C-29), 116.93 (C-34), 119.18 (C-32, 36), 122.18 (C-6'), 126.58 (C-4'), 132.39 (C-33, 35), 135.71 (C-5'), 138.58 (C-31), 143.10 (C-2'), 149.58 (C-3'), 150.09 (C-20), 174.14 (C-28), 217.72 (C-3); IR, cm^{-1} , ν : 1703 (C=O), 2219 (C≡C). Anal. Calcd for $\text{C}_{43}\text{H}_{54}\text{N}_2\text{O}_2$: C, 81.80; H, 8.43; N, 4.38. Found: C, 81.80; H, 8.62; N, 4.44.

3.6. N-(3-Oxolup-20(29)en-28-oyl)-4-iodoaniline 6

0.92 g (4.20 mmol) of *p*-iodoaniline, 4 mL of triethylamine and 2.0 g (4.2 mmol) of chloroanhydride **2** were added consecutively to 15 mL of dry benzene upon stirring. Reaction was then heated to 80 °C for 16 h. The reaction mixture was filtered through alumina (2 \times 2.5 cm). Evaporation of the solvent in vacuo provided 1.4 g (50%) of the product, mp 163–164 °C (benzene). ¹H NMR (CDCl_3 , 300.13 MHz) δ 0.89 (3H, s, Me-25), 0.94 (3H, s, Me-24), 0.98 (6H, s, Me-26, 27), 1.03 (3H, s, Me-23), 1.67 (3H, s, Me-30), 3.13 (1H, dt, 19, $J_1 = 4$ Hz, $J_2 = 11$ Hz), 4.58 (1H, s, 29), 4.72 (1H, s, 29), 7.26 (2H, m, 32, 36), 7.58 (2H, m, 33, 35); ¹³C NMR δ 14.38 (C-27), 15.68 (C-26), 15.77 (C-25), 19.32 (C-30), 19.43 (C-6), 20.82 (C-24), 21.27 (C-11), 25.45 (C-12), 26.38 (C-23), 29.39 (C-21), 30.59 (C-15), 33.53 (C-16), 33.63 (C-7), 33.93 (C-2), 36.76 (C-22), 37.49 (C-13), 37.84 (C-10), 39.46 (C-1), 40.55 (C-8), 42.43 (C-14), 46.27 (C-19), 47.13 (C-4), 49.86 (C-18), 50.07 (C-9), 54.92 (C-5), 56.45 (C-17), 86.76 (C-34), 109.42 (C-29), 121.75 (C-32, 36), 137.66 (C-33, 35), 137.89 (C-31), 150.30 (C-20), 174.31 (C-28), 217.73 (C-3); IR, cm^{-1} , ν : 1694 (C=O), 3443 (NH); Anal. Calcd for $\text{C}_{36}\text{H}_{50}\text{INO}_2$: C, 66.61; H, 8.09; N, 1.91. Found: C, 65.98; H, 7.69; N, 2.14.

3.7. N-(3-Oxolup-20(29)en-28-oyl)-4-(5-hydroxy-5-methylhexa-1,3-diyanyl)aniline 4

130 mg (0.198 mmol) of iodide **6**, 6 mg (0.03 mmol) of CuI, 10 mg (0.01 mmol) of $\text{PdCl}_2(\text{PPh}_3)_2$, 2 mL of triethyl amine and 33 mg (0.27 mmol) of diacetylene alcohol were added to 8 mL of

toluene under argon and upon stirring. The reaction mixture was kept at 55 °C for 12 h. At the end of the reaction, 10 mL of toluene was added. The reaction mixture was washed with aqueous ammonia. The organic layer was dried over Na₂SO₄, filtered and concentrated in vacuo. The product was precipitated by hexane to afford 72 mg (57%) of compound **4**, mp 183–184 °C (benzene). Physical and spectral properties of this compound are identical to those for the compound produced in the Cadiot–Chodkiewicz reaction (see above, mp 183–184 °C).

3.8. Biochemical experiments

The experiments were performed on outbred male mice, weighing between 22 and 25 g, obtained from the vivarium of Institute of Cytology and Genetics of Siberian Branch of Russian Academy of Sciences. During all experiments the animals were given granulated food and water ad libitum and kept under standard conditions. All studies were carried out in accordance with the Guidelines for the Care and Use of Laboratory Animals.

3.9. The CCl₄-induced hepatitis model

Acute toxic hepatitis was induced through a single oral administration of 25% CCl₄ in sunflower oil. Animals received the tested compounds orally as a water–Tween-80 suspension in the 50 mg/kg bw (**3b** and **3a**) and 20 mg/kg (**6**) doses 1 h before the hepatitis induction. The choice of the dose was based on the analysis of toxico-pharmacological properties of compounds predicted using the PASS software.²² A known antioxidant, dihydroquercetin (99% purity) was used as a reference compound in the effective oral dose of 100 mg/g. The control group was given an equivalent volume of the water–Tween suspension. Each group contained at least ten animals. The activity of alkaline phosphatase (ALP), alanine- and aspartate aminotransferases (ALT and AST) in the blood serum was determined the next day using standard reagent kits ('Biocon', 'Olvex Diagnosticum'). The level of thiobarbituric acid-reactive substances, TBARS, was determined using the well-established procedure.²³

3.10. Carrageenan-induced edema model

Inflammatory edema was induced by subplanar injection of 0.05 ml 1.5% carrageenan in aqueous–Tween suspension into the hind paw of 48 male mice. The test compounds were administered orally in a dose of 20 mg/kg (aqueous–Tween suspension) one hour before the phlogogenic agent introduction. The reference agent indomethacin ('Fluka') was administered in the same manner and in the same dose. The control group of animals received an equivalent portion of water–Tween mixture. The animals were sacrificed by cervical dislocation 5 h after the phlogogenic agent injection, the mouse paws were cut off at the ankle joint and weighed. The ratio of the difference in weight between the treated and untreated hind paws to the weight of the untreated hind paw was used as an index of paw edema. The anti-inflammatory effect was calculated as the decrease in the index of edema in comparison with the control group. The results were analyzed using STATISTICA 6 software. The differences were significant at $p < 0.05$.

Acknowledgments

This work was supported by the Interdisciplinary Grant No. 93 of SB of the Russian Academy of Sciences (2009–2011), Grant RFBR

No. 07-03-00048a, Grant 5.9.3. of the Russian Academy of Sciences (2009–2011) and the Chemical Service Centre of SB RAS. Work at FSU is supported by supported by the National Science Foundation (CHE-0848686).

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