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Original article

Synthesis, anti-HCV, antioxidant, and peroxynitrite inhibitory activity of fused benzosuberone derivatives

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ABSTRACT

Reaction of benzosuberone **1** with dimethylformamide–dimethylacetal (DMF–DMA) gives 2-dimethylamino-methylenebenozosuberone **2** which in turn reacts with heterocyclic amines to furnish new heterocyclic ring systems **6**–**9**. Moreover, enaminone **2** reacts with hydrazine hydrate and hydroxylamine hydrochloride to afford the corresponding benzo[6,7]cyclohepta[1,2–*c*]pyrazole (**10**) and benzo[6,7]cyclohepta[2,1–*d*]isoxazole (**12**), respectively. In addition, the reactions of enaminone **2** with active methylene compounds afforded benzo[6,7]cyclohepta[1,2–*b*]pyridines (**13**–**18**). The X-ray crystallographic analysis of compounds **6** and **16**, were recorded. We demonstrated the ability of nine new synthesized compounds to inhibit Hepatitis C Virus (HCV) and Subacute Sclerosing Panencephalitis (SSPE) due to structural similarity between ribavirin and some of the newly synthesized compounds were they contain triazoles and its bioisosters. In addition, the ability of ten synthesized compounds to react with the biologically relevant reactive nitrogen species, peroxynitrite was investigated indirectly by measurement of their ability to inhibit $ONOO^-$-induced tyrosine nitration. The antioxidant activity of these ten compounds was also studied using 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay.

1. Introduction

Enaminones are versatile reagents and their utility in heterocyclic synthesis has recently earned considerable attention [1–4]. Benzosuberone derivatives are known as cytotoxic and anticancer agents [5–8]. In this paper, we continue with our interest in the development of new and simple methods for the synthesis of substituted heterocycles from enaminone with multiplet functional groups with anticipated biological activity [2]. Thus we used the enaminone **2** namely, 2-dimethylaminomethylene benzosuberone in the synthesis of a variety of novel fused benzosuberone and investigated their anti-HCV activities. In addition, the 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging method has been used to measure the antioxidant property of the newly synthetic compounds. These compounds were also examined for their ability to protect against peroxynitrite-dependent nitration reactions.

2. Chemistry

Benzosuberone **1** reacts with dimethylformamide–dimethylacetal (DMF–DMA) and furnished a single product (examined by TLC) that was identified as 2-dimethylamino-methylenebenzosuberone **2** (Scheme 1). Elemental analyses and spectral data were in complete accordance with the assigned structure **2**. For example, the ^{1}H NMR spectrum of compound **2** revealed two singlet signals at δ 3.07 and 7.58 ppm characteristic for *N*,*N*-dimethylamino and the exocyclic C=CH protons, respectively [9]. The enaminone **2** was assigned the *E*-configuration **2E** based on their ^{1}H NMR spectra which revealed that the exocyclic C=CH proton signal at δ 7.58 ppm correlated for *E*-isomers. On the other hand, *Z*-isomer **2*Z*** of analogous structures was reported to appear at δ 6.9 ppm [10].

As previously reported enominones can be used as potential precursors for fused heterocyclic systems when reacting with heterocyclic amines [11]. Thus, treatment of enaminone **2** with 3-aminotriazole **3**, 4-phenyl-3-aminopyrazole **4**, and 2-aminobenzimidazole **5** in refluxing acetic acid gave three new ring systems namely 9,10,11-trihydrobenzo[6′,7′]cyclohepta[2′,1′–*e*]triazolo[2,3–*a*] pyrimidine (**6**), 9,10,11-trihydrobenzo[6′,7′]cyclohepta[2′,1′–

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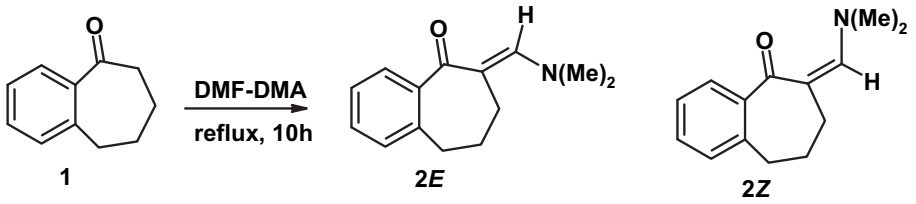
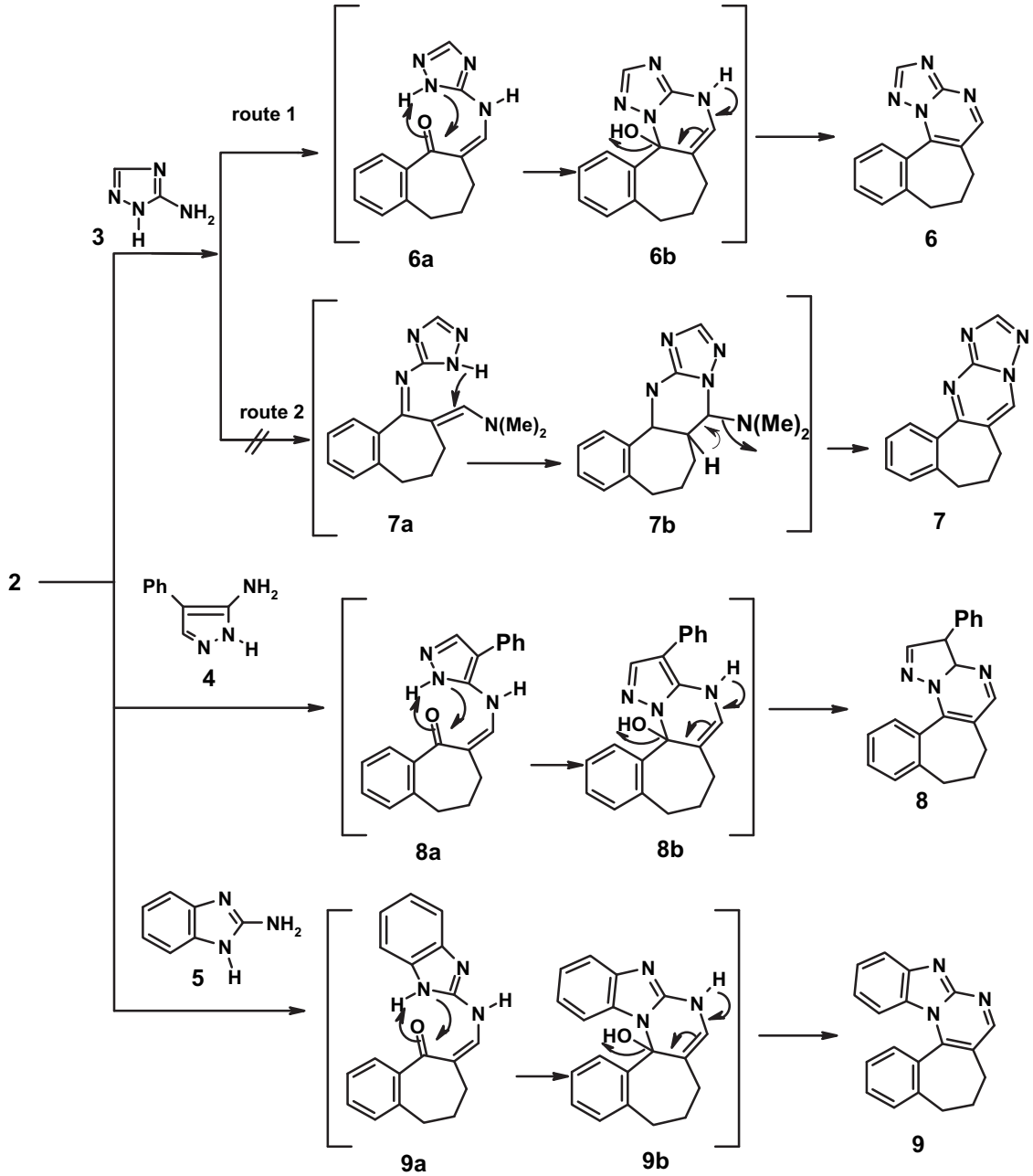


Scheme 1. Synthesis of enaminone **2**.

e]pyrazolo[2,3–*a*]pyrimidine (**8**) and 9,10,11-trihydrobenzo[6′,7′]-cyclohepta[2′,1′-*e*]pyrimido [1,2-*a*]benzimidazole (**9**), respectively (Scheme 2). Condensation of 3-amino-[1,2,4]triazole (**3**) with enaminone **2** can proceed in two possible ways, as shown in Scheme 2. In route 1, the nucleophilic amino group attacks the double bond *via* a Michael addition reaction, followed by elimination of the dimethylamino group. Cyclization of intermediate **6a** with concurrent dehydration of **6b** gave compound **6**. On the other hand, in route 2, the exocyclic amino group of triazole attacks the carbonyl group to generate isomeric structure **7** *via* intermediate **7a** and **7b**. This



Scheme 2. Reaction of enaminone **2** with heterocyclic amines.

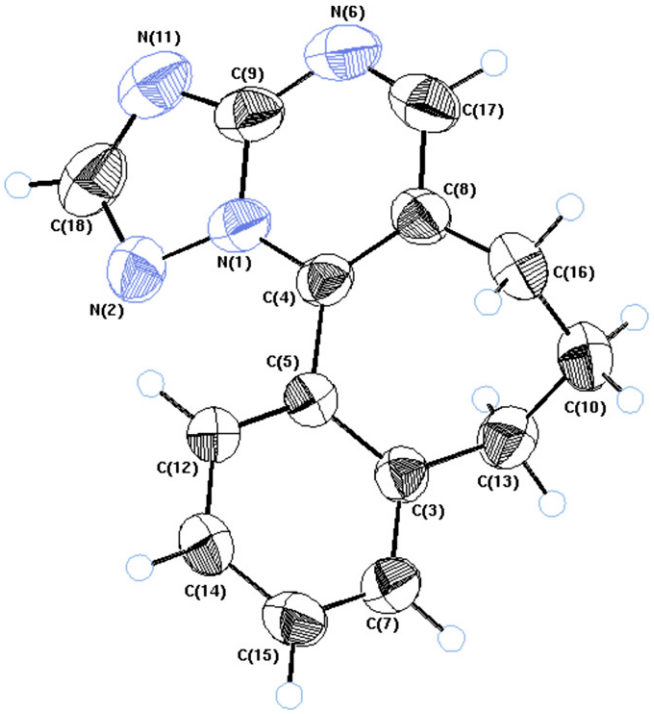


Fig. 1. Crystal structure of compound **6.**

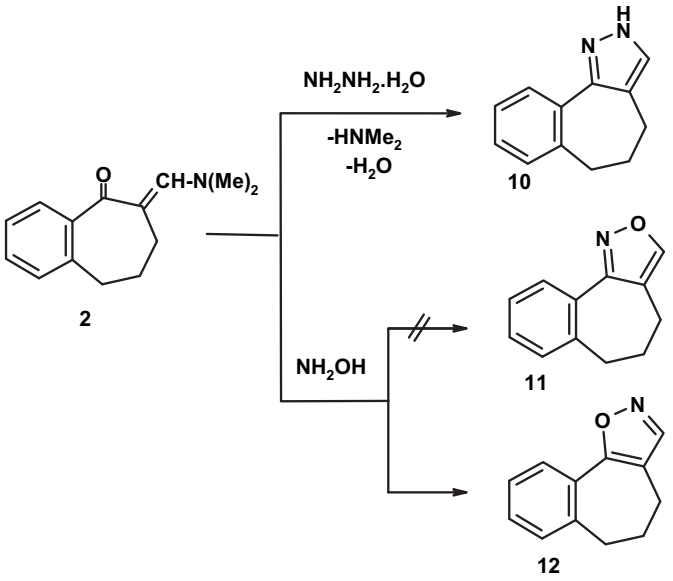
regioselectivity has been unambiguously substantiated through the X-ray crystallographic analysis of the product **6** rather than product **7** as shown in Fig. 1. Typical bond lengths and bond angles are shown in Table 1.

Reaction of enaminone **2** with hydrazine hydrate in absolute ethanol resulted in the formation of a single product **10** as examined by TLC (Scheme 3). Mass spectrum and elemental analysis showed that the reaction product **10** has the molecular formula $C_{12}H_{12}N_2$ with molecular ion peak at 184. ^{1}H NMR spectrum, showed two characteristic singlet signals at δ 7.47 and 12.40 ppm for pyrazole-H and NH [12–14]. It is assumed that compound **10** is formed *via* initial addition of the amino group in hydrazine to the enamine double bond followed by elimination of dimethylamine and water to give the final isolable product **10**. Similarly, enaminone **2** reacts with hydroxylamine hydrochloride in absolute ethanol in the presence of anhydrous sodium acetate to give one isolable product, namely, benzo[6,7]cyclohepta[2,1–*d*]isoxazole **12** rather than isomeric form benzo[6,7]cyclohepta[1,2–*c*]isoxazole **11** (Scheme 3). Structure **12** was assigned as the correct structure on the basis of its ^{1}H NMR spectrum where a resonance for H-3 of isoxazole appeared typically at $\delta = 8.50$ ppm. The isomeric product was ruled out as H-5 of isoxazole would be expected to resonate at lower field around at δ 9.20 ppm [15,16].

Reaction of enaminone **2** with acetylacetone and ethyl acetoacetate in glacial acetic acid in the presence of ammonium acetate gave benzo[6,7]cyclohepta [1,2–*b*]pyridine derivatives **13** and **14**,

Table 1
Selected bond angles and bond lengths of compound **6**.

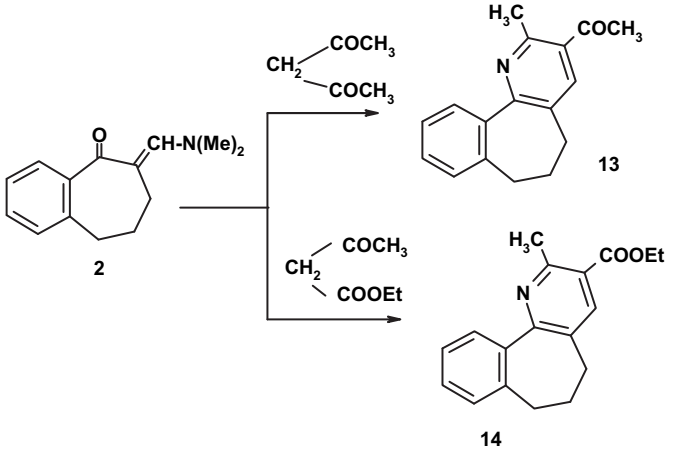
Bond	Angle	Bond	Lenghth (Å)
N2–N1–C4	127.5	N1–N2	1.372
N1–N2–C18	100.5	N2–C18	1.331
C9–N11–C18	102.4	C9–N11	1.329
C9–N6–C17	116.0	N6–C17	1.310
C4–C8–C16	120.3	C4–C8	1.3



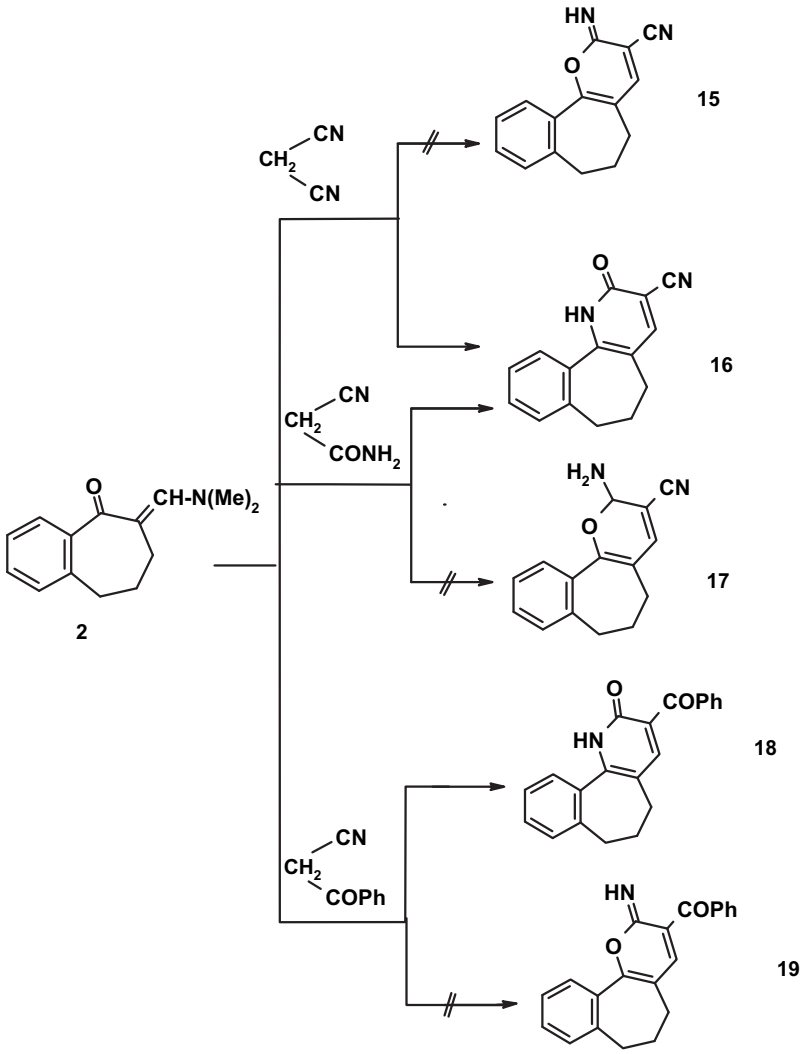
Scheme 3. Reaction of enaminone **2** with hydrazine hydrate and hydroxyl amine.

respectively (Scheme 4). The structure of the products were assigned based on the ^{1}H NMR which shows a characteristic singlet signals at δ 8.14 and 8.05 ppm characteristic for the pyridine-H of compounds **13** and **14**, respectively [17].

Two possible structures 2-iminobenzo[6,7]cyclohepta[1,2–*b*]pyran-3-carbonitrile (**15**) or 1,2-dihydro-2-oxo-benzo[6,7]cyclohepta[1,2–*b*]pyridine-3-carbonitrile (**16**) have been proposed for the reaction of enaminone **2** with malononitrile in refluxing ethanolic sodium ethoxide (Scheme 5). Structure **15** was ruled out based on ^{1}H NMR and IR spectral data. For example, ^{1}H NMR spectrum of the product, showed the absence of imine signal at δ 3.07 ppm. Also, IR spectrum revealed the amidic carbonyl absorption band at $\upsilon = 1650$ cm^{-1}. Furthermore, the product **16** was substantiated by independent synthesis from cyanoacetamide and enaminone **2**. The two reaction products were identical in all spectral data (see Experimental). Also, the X-ray crystal structure indicated that the reaction of enaminone **2** with each of malononitrile and cyanoacetamide gave the same structure **16** as shown in Fig. 2. In Table 2, selected bond angles and bond lengths are reported. In a similar manner, the enaminone **2** was reacted with ω-cyanoacetophenone in acetic acid in the presence of ammonium acetate to



Scheme 4. Reaction of enaminone **2** with active methylene compounds.



Scheme 5. Reaction of enaminone **2** with active methylene nitrile compounds.

give compound **18** (Scheme 5). 3-Benzoyl-5,6,7-trihydrobenzo[6,7] cyclohepta[1,2-b]pyridine-2(1H)-one (**18**) was assigned as a reaction product based on correct spectral and analytical data (see Experimental).

In continuation of our work, benzosuberone was allowed to react with different enaminones **20a–c** to give 5,6,7-trihydrobenzo[6,7]cyclohepta[1,2-b]pyridine derivatives **21a–c** (Scheme 6). The spectral data were found to be in complete agreement with the proposed structures (see Experimental).

3. Pharmacology

Benzosuberone derivatives have some cytotoxic,antioxidant and anti angiogenic activities [18] so depending on this strong biological rational we aiming at test the antioxidant activities, this together with testing the anti –HCV activities based on the structural similarity between our end products and ribavirin wer the triazole and its bioisosters presents.The obtained results prompted us to investigate the anti SS PE activities where it need special structural features in drugs to be act on this highly resistant viral category ,we though this due to the prescence of triazole or two heteroatom five membed or one heteroatom six membered ring containg electron deficient groups fused to the bezosuberone.

3.1. Results and discussion

3.1.1. Hepatitis C virus (HCV) NS3-4A protease inhibitory activities in both HCV replicon cells and in hamster brains

In order to monitor the potential of nine new synthesized compounds to inhibit HCV we used two methods for determination of minimum inhibitory concentration (MIC) of these compounds. Firstly, their antiviral activities in HCV Replicon Cells was measured by their Hepatitis C Virus (HCV) NS3-4A Protease Inhibitory activities. Secondly, their antiviral chemotherapy for Subacute Sclerosing Panencephalitis (SSPE) were determined in Hamster Brains. Ribaverin was used in both methods as positive control.

Table 3 showed the MIC values for the tested compounds and ribaverin against HCV and SSPE. The results showed that all the nine tested compounds are highly effective at very low concentration compared to ribaverin in the following order: **21a** > **6** > **14** > **16** > **9** > **21b** > **10** > **13** > **12** These results indicate that these compounds are also very potent inhibitors of HCV in both cases.

3.1.2. DPPH radical scavenging activity

Radical scavenging activities of compounds **2**, **6**, **9**, **10**, **12–14**, **16**, **21a**,**b** were measured using model colourimetric test: DPPH radical

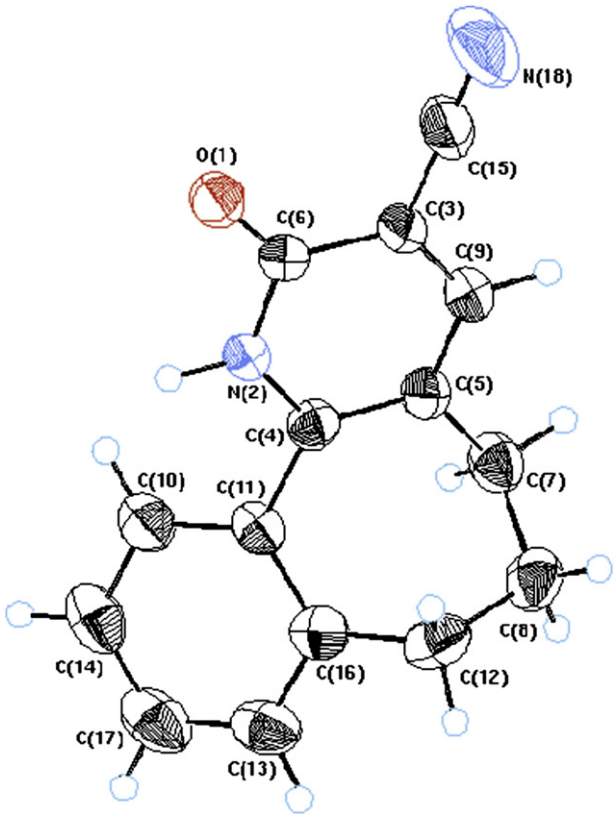


Fig. 2. Crystal structure of compound **16.**

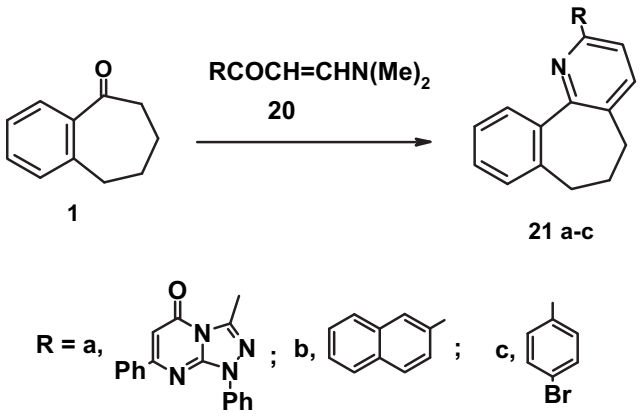
scavenging test. The results are summarized in Table 3. The stable radical DPPH has been used widely for the determination of primary antioxidant activity, that is, the free radical scavenging activities of pure antioxidant compounds, plant and fruit extracts and food materials. The assay is based on the reduction of DPPH radicals in methanol which causes an absorbance drop at 515 nm. Among the ten compounds tested for antioxidant activity using DPPH method six of the tested compounds exhibited good scavenging activity against the DPPH radical in the following order: **2** > **14** > **21b** > **10** > **12** > **21a** (Table 4). The values were found to be less than that of standard ascorbic acid. The other four compounds were considerably less effective radical scavengers in the order **13** > **9** > **6** > **16** (Table 4).

The mechanism of antiviral activity is due to enhance host T-cell-mediated immunity against viral infection through helping to switch the host T-cell phenotype from type 2 to type 1. Also the mechanism of antiviral activity is due to transient increase in the mutation rate of HCV lethal mutagenesis and error catastrophe is unlikely to be the mechanism of SSPE during treatment of CHC.

Te antioxidant activities of the newly synthesize compounds is mainly due to the free scavenging activities of the benzosuberone ring and heterocyclic ring system

Table 2
Selected bond angles and bond lengths of compound **16**.

Bond	Angle	Bond	Length (Å)
C4–N2–C6	125.3	N2–C4	1.371
C6–C3–C9	121.1	N2–C6	1.374
C6–C3–C15	117.6	C3–C6	1.340
C9–C3–C15	121.3	C3–C9	1.372
N2–C4–C5	119.7	C4–C5	1.376
C3–C15–N18	179.8	C3–C15	1.429
O1–C6–N2	121.0	O1–C6	1.246



Scheme 6. Reaction of benzosuberone with enaminone **2**.

3.1.3. Inhibition of peroxynitrite-induced tyrosine nitration

The reactive nitrogen species peroxynitrite ($ONOO^-$) has been implicated in numerous human disease pathologies and its role is usually inferred from the measurement of 3-nitrotyrosine (3-NT). Peroxynitrite and species derived from it can oxidise and nitrate lipids [19], proteins [20], DNA [21,22] and carbohydrates [23] leading to tissue damage in a number of pathological conditions in humans and in experimental animals. Due to the cytotoxicity of $ONOO^-$ and its apparent formation at sites of tissue injury [24,25], there has been considerable interest in the ability of natural and synthetic antioxidants to diminish $ONOO^-$ damage. In the present work, the ability of ten synthetic compounds to prevent $ONOO^-$-mediated damage was examined using the model system: $ONOO^-$-mediated tyrosine nitration. When the amino acid tyrosine is exposed to $ONOO^-$ at pH 7.4, 3-NT is formed [26]. Fig. 3A and B show that the addition of the ten synthesized compounds, significantly inhibited tyrosine from $ONOO^-$-mediated nitration in a dose dependent matter. The relative inhibitory activity of these compounds is summarized in Table 5 as IC_{50} values. Under our experimental conditions the relative potencies of the ten compounds were **2** > **12** > **13** > **21b** > **10** > **16** > **6** > **14** > **9** > **21a**.

4. Conclusion

In conclusion, enaminone **2** was used as precursor for the synthesis of a variety of heterocyclic ring systems formed upon reaction with heterocyclic amines, hydrazine hydrate, hydroxyl amine and active methylene to afford heterocyclic derivatives with high biological activity. The structures of the newly synthesized compounds were confirmed by spectral data and elemental

Table 3
MIC of Ribaverin and the nine tested compounds against HCV and SSPE.

Tested Compound%	MIC μg/ml	
	HCV	Subacute Sclerosing Panencephalitis (SSPE)
Ribaverin	16.15	**77.89**
12	7.66	**51.11**
13	7.09	**41.12**
10	6.22	**40.15**
21b	5.98	**38.40**
9	6.00	**34.89**
16	4.45	**23.01**
14	3.58	**12.55**
6	2.21	**11.12**
21a	**1.15**	**7.56**

Table 4
Decrease of DPPH absorbance (%) by ten synthesized compounds.

Compound No.	Decrease of DPPH absorbance% mean ± SD ($n = 3$)
2	57.883 ± 3.961
6	27.701 ± 3.094
9	28.175 ± 5.105
10	45.369 ± 2.017
12	44.119 ± 6.12
13	37.209 ± 4.094
14	54.436 ± 5.598
16	27.390 ± 7.013
21a	39.994 ± 7.91
21b	54.199 ± 6.537
Ascorbic acid (standard)	79.042 ± 2.928

analyses and also, the structure of two compounds were confermed by X-ray analyses. The studies described here demonstrate the ability of nine new synthesized compounds to inhibit HCV at very low concentration. In addition, ten of the newly synthesized compounds were tested for antioxidant activity using DPPH method, six of the tested compounds exhibited good scavenging activity against the DPPH radical in the following order: **2** > **14** > **21b** > **10** > **12** > **21a**. The values were found to be less than that of standard ascorbic acid. The other four compounds were considerably less effective radical scavengers in the order **13** > **9** > **6** > **16**. Also, the ability of ten synthetic compounds to prevent $ONOO^-$-mediated damage was examined using the model system: $ONOO^-$-mediated tyrosine nitration which indicated that the relative potencies of the ten compounds were **2** > **12** > **13** > **21b** > **10** > **16** > **6** > **14** > **9** > **21a**.

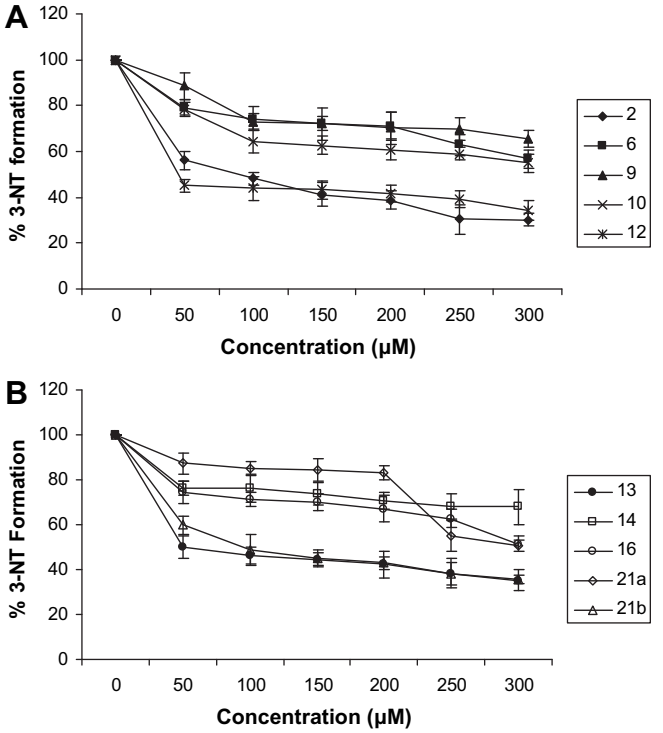


Fig. 3. Inhibition of tyrosine nitration by ten compounds. Tyrosine (100 μM) and $ONOO^-$ (100 μM) incubated in phosphate buffer (100 mM), pH 7.4 at 37 °C for 15 min ± compounds (0–300 μM). 3-NT formed was measured by HPLC as described under Materials and Methods. Data are mean ± SD of 3 independent experiments. Control = $ONOO^-$ + tyrosine, (A) for compounds **2**, **6**, **9**, **10** and **12**, (B) for compounds **13**, **14**, **16**, **21a** and **21b.**

5. Experimental protocol

5.1. Chemistry

All melting points were determined on an electrothermal Gallenkamp apparatus. Solvents were distilled and dried by standard literature procedures prior to use. The IR spectra were measured on a Pye-Unicam SP300 instrument in potassium bromide discs. The ^1H NMR spectra were recorded on a Varian Mercury VXR-300 MHz spectrometer and the chemical shifts δ (ppm) down field from tetramethylsilane (TMS) as an internal standard. The mass spectra were recorded on a GCMS-Q1000-EX Shimadzu and GCMS 5988-A HP spectrometers, the ionizing voltage was 70 eV. Elemental analyses were carried out by the Microanalytical Center of Cairo University, Giza, Egypt. Tyrosine, 3-nitrotyrosine (3-NT) and 1,1-Diphenyl-2-picryl hydrazyl (DPPH) were obtained from Sigma–Aldrich (St. Louis, U.S.A).

5.2. Crystallographic analysis

The crystals were mounted on a glass fiber. All measurements were performed on an ENRAF NONIUS FR 590. The data were collected at a temperature of 25 °C using the ω scanning technique to a maximum of a 20 of 22.986°. The structure was solved by direct method using SIR 92 and refined by full-matrix least squares. Non-hydrogen atoms were refined anisotropically. Hydrogen atoms were located geometrically and were refined isotropically.

5.2.1. Crystal data

For compound **6**: $C_{14}H_{12}N_4$, $M = 236.28$, monoclinic, $a = 7.8943$ (7), $b = 17.640$ (2), $c = 8.2084$ (9) A°, $v = 1134.5$ (2), $\alpha = \gamma = 90.00°$, $\beta = 97.020$ (4), space group: $P2_1/c$, $Z = 4$, $D_x = 1.383$ Mg m^{-3} reflection 2069 measured, $\theta_{max} = 21.50°$. Fig. 1 illustrates the structure as determined.

For compound **16**: $C_{15}H_{12}N_2O$, $M = 236.27$, monoclinic, $a = 8.1328$ (4), $b = 9.2799$ (4), $c = 16.2253$ (8) Å, $v = 1220.75$ (10), $\alpha = \gamma = 90.00°$, $\beta = 94.512$ (2), space group: $P2_1/c$, $Z = 4$, $D_x = 1.286$ Mg m^{-3} reflection 2968 measured, $\theta_{max} = 22.98°$. Fig. 2 illustrates the structure as determined. Full data can be obtained on request from the CCDC [27].

5.3. Preparation of 2-dimethylaminomethylene-1-benzosuberone (*2*)

A mixture of compound **1** (0.01 mol, 1.6 g) and DMF–DMF (2 ml) was heated under reflux for 10 h. The reaction mixture was triturated with ethanol to give a solid product that was collected by

Table 5
IC50 values of the compounds tested for the inhibition of $ONOO^-$-mediated 3-nitrotyrosine formation.

Compound No.	IC50 (μM) mean ± SD ($n = 3$)
2	146.316 ± 9.998
6	315.052 ± 19.709
9	341.659 ± 20.864
10	277.021 ± 17.836
12	148.771 ± 10.197
13	157.659 ± 10.986
14	328.935 ± 20.575
16	290.664 ± 18.886
21a	349.175 ± 21.264
21b	169.929 ± 11.771
Trolox	58.430 ± 5.9

filtration and crystallized from ethanol to give compound **2** as deep orange crystals, yield (95%) m.p 85–86 °C [28], ^{1}H NMR (DMSO-d_6) at δ 1.74–2.66 (m, 6H, $3CH_2$), 3.07 (s, 6H, $2CH_3$), 7.13–7.44 (m, 4H, ArH), 7.58 (s, 1H, =CH); IR 1720 cm^{-1} MS *m*/*z* (%) 217 ($M^+ + 2$, 15), 216 ($M^+ + 1100$), 215 (M^+, 53), 198 (26), 84(5), 77(2). Anal. Calcd. For $C_{14}H_{17}NO$ (215.30) C, 78.10; H, 7.96; N, 6.51. Found C, 77.93; H, 7.82; N, 6.30%.

5.4. Reaction of enaminone 2 with heterocyclic amines

To a solution of **2** (0.005 mol, 1.08 g) in acetic acid (20 ml) was added (0.005 mol) of the appropriate heterocyclic amines (**3**–**5**). The mixture was heated under reflux for 6 h. The solid deposited after cooling was then collected by filtration and crystallized from the appropriate solvent. The physical constants together with the spectral data of products **6**, **8**, **9** are depicted below.

5.4.1. 9,10,11-Trihydrobenzo[6′,7′]cyclohepta[2′,1′-e]triazolo[2,3-a] pyrimidine (*6*)

Golden yellow crystals yield (69%), m.p > 300 °C. (Ethanol) ^{1}H NMR (DMSO-d_6) 1.70–2.66 (m, 6H, $3CH_2$), 7.47–8.08 (m, 4H, ArH), 8.62 (s, 1H, pyrimidine-H), 8.88 (s, H, triazole-H). MS *m*/*z* (%) 237 ($M^+ + 1$, 16), 236 (M^+, 100), 235 (59), 128 (11), 115 (21), 89 (14), 77 (15). Anal. Calcd. for $C_{14}H_{12}N_4$ (236.28) C, 71.17; H, 5.12; N, 23.71. Found C, 71.20; H, 4.99; N, 23.54%.

5.4.2. 1-Phenyl-9,10,11-trihydrobenzo[6′,7′]cyclohepta[2′,1′-e]pyrazolo[2,3-a] pyrimidine (*8*)

Golden yellow crystals, yield (72%) m.p > 300 °C. (Dioxan/Ethanol). ^{1}H NMR (DMSO-d_6) at δ 1.60–2.73 (m, 6H, 3 CH_2); 7.15–8.23 (m, 9H, ArH); 8.54 (s, 1H, pyrimidine-H); 8.74 (s, 1H, pyrazole-H). MS *m*/*z* (%) 312 ($M^+ + 1$,36), 311 (M^+, 100), 310(88). 285(14), 283 (14), 255(20), 238(17), 180 (10), 142 (21), 141 (25), 124 (11), 115 (13), 77 (18). Anal. Calcd. For $C_{21}H_{17}N_3$ (311.39) C, 81.00; H, 5.50; N, 13.49. Found C, 80.90; H, 5.31; N, 13.41%.

5.4.3. 9,10,11-Trihydrobenzo[6′,7′]cyclohepta[2′,1′-e]pyrimido[1,2-a] benzimidazole (*9*)

Yellow crystals, yield (60%), m.p 226 °C (Dioxan/Ethanol). ^{1}H NMR (DMSO-d_6) at δ 1.95–2.84 (m, 6H, $3CH_2$); 7.09–7.88 (m, 8H, ArH); 8.78 (s, 1H, pyrimidine-H). MS *m*/*z* (%) 287 ($M^+ + 2$, 3), 286 ($M^+ + 1$, 24), 285 (M^+, 100), 284 (43), 270 (8), 257 (10), 135 (3), 115 (4), 102 (3), 90 (3), 77 (4). Anal. Calcd. For $C_{19}H_{15}N_3$ (285.35) C, 79.98; H, 5.30; N, 14.72. Found C, 79.90; H, 5.08; N, 14.52%.

5.5. Synthesis of 4,5,6-trihydro-2H-benzo[6,7] cyclohepta[1,2-c]pyrazole (*10*)

To a solution of **2** (0.005 mol, 1.08 g) in absolute ethanol (20 ml) was added (0.005 mol, 0.25 g) of hydrazine hydrate. The mixture was then heated under reflux for 2 h. The precipitate formed was collected by filtration and crystallized from ethanol as pale yellow crystals yield (78%) m.p 76 °C [29], ^{1}H NMR (DMSO-d_6) at δ 1.74–2.85(m, 6H, $3CH_2$), 7.17–7.27 (m, 4H, ArH), 7.47 (s, 1H, pyrazole-H), 12.60 (s, 1H, NH). IR 3143 cm^{-1} MS *m*/*z* (%) 185 ($M^+ + 1$, 17) 184 (M^+, 100), 169 (27), 156 (36), 128 (26), 115 (28), 102 (12), 91 (13), 77 (20).Anal. Calcd. For $C_{12}H_{12}N_2$ (184.24) C, 78.23; H, 6.57; N, 15.20. Found C, 78.48; H, 6.40; N, 15.00%.

5.6. Synthesis of 4,5,6-trihydro-2H-benzo[6,7]cyclohepta[2,1-d]isoxazole (*12*)

To a solution of **2** (0.005 mol, 1.08 g) in absolute ethanol was added (0.005 mol, 0.35 g) of hydroxylamine hydrochloride in the presence of anhydrous sodium acetate (0.005 mol, 0.41 g). The reaction mixture was then heated under reflux for 5 h. The solid formed was collected by filtration and crystallized from Dioxan/Ethanol to give white solid, yield (70%), m.p > 300 °C, ^{1}H NMR (DMSO-d_6) 1.91–2.91 (m, 6H, 3 CH_2) 7.30–7.37 (m, 4H, ArH), 8.50 (s, 1H, isoxazole-H), MS *m*/*z*(%) 186 ($M^+ + 1$, 91), 185 (M^+, 100), 158 (21), 157 (65), 156 (82), 126 (32), 125 (94), 116 (47), 77(29). Anal. Calcd. For $C_{12}H_{11}NO$ (185.23) C, 77.81; H, 5.99; N, 7.56. Formed C, 77.63; H, 5.69; N, 7.42%.

5.7. Preparation of compounds *13* & *14*

To a solution of **2** (0.005 mol, 1.08 g) in acetic glacial in the presence of ammonium acetate (0.5 g), was added acetylacetone or ethyl acetoacetate (0.005 mol). The reaction mixture was heated under reflux for several hours. The reaction was followed by TLC. The solvent was evaporated under reduced pressure and the oil residue was triturated with ethanol to give the solid products **13** and **14,** respectively.

5.7.1. 3-Acetyl-2-methyl-5,6,7- trihydrobenzo[6,7]cyclohepta[1,2–b] pyridine (*13*)

Yellow solid, yield (60%); m.p 126–127 °C (Ethanol) ^{1}H NMR (DMSO-d_6) 1.71–2.45 (m, 6H, $3CH_2$), 2.62 (s, 3H, CH_3), 2.67 (s, 3H, CH_3), 7.30–7.65 (m, 4H, ArH), 8.14 (s, 1H, pyridine-H); IR 1678 (C=O) cm^{-1} MS *m*/*z* (%) 252 ($M^+ + 1$, 16), 251 (M^+, 52), 250 (25), 236 (100), 235 (20), 206 (18), 189 (15), 166 (30), 103 (36), 77 (41). Anal. Calcd. For $C_{17}H_{17}NO$ (251.33) C, 81.24; H, 6.82; N, 5.57. Found. C, 81.21; H, 6.53; N, 5.30%.

5.7.2. Ethyl-2-methyl-5,6,7–trihydrobenzo[6,7]cyclohepta[1,2–b]pyridine-3-carboxylate (*14*)

Pale yellow solid, yield (62%); m.p. 154–156 °C (Ethanol). ^{1}H NMR (DMSO-d_6) 1.25 (*t*, $J = 7$ Hz, 3H, CH_3), 1.87–2.76 (m, 6H, $3CH_2$), 2.42 (s, 3H, CH_3), 4.39 (q, $J = 7$ Hz, 2H, CH_2), 7.26–7.63 (m, 4H, ArH), 8.05 (s, 1H, Pyridine H). IR 1710 cm^{-1} MS *m*/*z* (%) 282 ($M^+ + 1$, 1), 281 (M^+, 5), 200 (12), 187 (15), 186 (24), 170 (29), 144 (26), 141 (43), 131 (51), 128 (100), 118 (17), 115 (83), 103 (67), 91 (92), 77 (49). Anal. Calcd. For $C_{18}H_{19}NO_2$ (281.36) C, 76.84; H, 6.81; N, 4.98. Found C, 76.62; H, 6.65; N, 4.69%.

5.8. 2-Oxo-2,5,6,7-tetrahydro-1H-benzo[6,7]cyclohepta[1,2-b]pyridine-3-carbonitrile (*16*)

To a solution of **2** (0.005 mol, 1.08 g) in ethanolic sodium ethoxide solution (0.12 g of sodium metal in 20 ml absolute ethanol) was added (0.005 mol) malononitrile or cyanoacetamide. The reaction mixture was heated under reflux for 5 h, and then poured into ice-cold water. The solution was acidified with diluted HCl and the solid deposited was collected by filtration and crystallized from dioxan/ethanol, yield (60%), m.p > 300 °C, ^{1}H NMR(DMSO-d_6) 2.04–2.56 (m, 6H, $3CH_2$), 7.36–7.53 (m, 4H, ArH), 8.12 (s, 1H, pyridine-H), 12.66 (s, 1H, NH). IR 3386, 2229, 1647 cm^{-1} MS *m*/*z* (%) 237 ($M^+ + 1$, 13), 236 (M^+, 100), 235 (23), 221 (28), 208 (10), 115 (2), 104 (5), 89 (4), 76 (5). Anal. Calcd. for $C_{15}H_{12}N_2O$ (236.28) C, 76.25; H, 5.12; N, 11.86. Found C, 76.05; H, 5.10; N, 11.62%

5.9. Synthesis of 3-benzoyl-5,6,7-trihydrobenzo[6′,7′]cyclohepta[1,2-b] pyridine-2(1H)-one (18)

To a solution of **2** (0.005 mol, 1.08 g) in ethanol (30 ml) containing piperidine (0.5 ml) as a catalyst, was added ω-cyanoacetophenone (0.005 mol, 0.71 g). The reaction mixture was heated under reflux for several hours and the progress of the reaction was monitored by TLC. The solvent was evaporated under reduced pressure, the oil residue was treated with methanol to give

compound **18** as buff crystals, yield (72%) m.p 105 °C (ethanol) ^{1}H NMR 1.73–2.66 (m, 6H, $3CH_2$), 7.05–8.00 (m, 9H, ArH), 8.24 (s, 1H, pyridine-H), 11.98 (s, 1H, NH). IR 3215, 1686, 1674 cm^{-1} MS *m*/*z* (%) 316 ($M^+ + 1$, 20), 315 (M^+, 46), 314 (32), 147 (34), 104 (65), 89 (20), 77 (100). Anal. Calcd. for $C_{21}H_{17}NO_2$ (315.38) C, 79.98; H, 5.43; N, 4.44. Found C, 79.75; H, 5.24; N, 4.12%

5.10. Preparation of compounds **21a–c**

To a solution of enaminones **20a–c** (0.005 mol) in acetic acid (20 ml) in the presence of ammonium acetate (0.5 g) was added benzosuberone **1** (0.005 mol, o.80 g). The reaction mixure was refluxed for 5 h., the solid formed was collected by filtration and crystallized from acetic acid to yield compounds **21a–c**.

5.10.1. 3-(6,7-Dihydro-5H-benzo[6,7]cyclohepta[1,2-b]pyridine-2-yl)-1,7-diphenyl-1H-[1,2,4]triazolo[4,3-a]pyrimidin-5-one (**21a**)

Yellow solid, yield (85%), m.p 200 °C, ^{1}H NMR (DMSO-d_6) δ 1.90–2.70 (m, 6H, $3CH_2$); 6.70 (s, 1H, Pyrimidine-H), 7.49–8.30 (m, 14H, ArH); 8.71 (d, $J = 8$ Hz, 2H, pyridine-H), 9.44 (d, $J = 8$ Hz, 2H, pyridine-H). MS *m*/*z* (%) 482 ($M^+ + 1$, 5), 481 (M^+, 11), 456 (19), 376 (22), 178 (34), 128 (37), 89 (65), 77 (100). IR 1685 cm^{-1}. Anal. calcd. For $C_{31}H_{23}N_5O$ (481.56) C, 77.32; H, 4.81; N, 14.54. Found C, 77.15; H, 4.50; N, 14.31%.

5.10.2. 2-(2-Naphthyl)-6,7-dihydro-5H-benzo[6,7]cyclohepta[1,2-b]pyridine (**21b**)

Pale yellow sheets, yield (85%), m.p 178–180 °C, ^{1}H NMR (DMSO-d_6) at δ 1.70–2.61 (m, 6H, $3CH_2$), 7.59–8.47 (m, 10H, ArH), 8.47 (d, $J = 8$ Hz, 2H, pyridine-H), 8.84 (s, 1H, naphthyl-H) 9.11 (d, $J = 8$ Hz, 2H, pyridine-H). MS *m*/*z* (%) 321 (M^+, 2), 316 (40), 202 (80), 157(66), 105 (100), 95 (87), 80 (67), 69 (80). Anal. Calcd. For $C_{24}H_{19}N$ (321.43) C, 89.68; H, 5.96; N, 4.36. Found C, 89.55; H,5.68; N, 4.21%.

5.10.3. 2-(4-Bromophenyl)-6,7-dihydro-5H-benzo[6,7]cyclohepta[1,2-b] pyridine (**21c**)

Golden yellow crystals, yield (82%), m.p 212 °C. ^{1}H NMR (DMSO-d_6) at δ 1.90–2.4 (m, 6H, $3CH_2$); 7.74–7.83 (m, 8H, ArH), 8.15 (d, $J = 8$ Hz, 2H, pyridine-H), 8.20 (d, $J = 8$ Hz, 2H, pyridine-H). MS *m*/*z* (%) 352 ($M^+ + 2$, 10), 351 ($M^+ + 1$, 15), 350 (M^+, 33), 270 (14), 193 (22), 157(80), 105 (64), 95 (87), 77 (100). Anal. Calcd. For $C_{20}H_{16}BrN$ (350.26) C,68.58; H,4.60; N,4.00. Found C, 68.32; H, 4.28; N,3.91%.

5.11. Pharmacology

5.11.1. Hepatitis C virus (HCV) NS3-4A protease inhibitory activities in HCV replicon cells

5.11.1.1. Determination of minimum inhibitory concentration (MIC), of ribavirin and different tested compounds in HCV replicon cells was performed as follow. Briefly, 1×10^4 replicon cells per well were plated in 96-well plates. On the following day, replicon cells were incubated at 37 °C for the indicated period of time with antiviral agents serially diluted in DMEM plus 2% FBS and 0.5% dimethyl sulfoxide (DMSO). Total cellular RNA was extracted using an RNeasy-96 kit (QIAGEN, Valencia, CA), and the copy number of HCV RNA was determined using a quantitative RTPCR (QRT-PCR) assay. Each datum point represents the average of five replicates in cell culture. The cytotoxicity of tested compound was measured under the same experimental settings using a tetrazolium (MTS)-based cell viability assay (Promega, Madison, WI). For the cytotoxicity assay with human hepatocyte cell lines, 1×10^4 parental Huh-7 cells per well or 4×10^4 HepG2 cells per well were used.

5.11.1.2. MIC of the tested compounds in Hamster Brains for antiviral chemotherapy for Subacute Sclerosing Panencephalitis (SSPE). Under ether anesthesia, 50 ml of ribavirin or tested compound solutions at dosages of 5, 10, and 20 mg/kg/day was injected for 10 days intracranially to a depth of 2 mm by using a 27-gauge needle and was placed within the subarachnoid space. At 1, 2, 3, 5, 7, 10, 12, 15, and 20 days after the initial injection, four hamsters from each group were sacrificed. The brains were aseptically removed, washed twice with phosphate-buffered saline (PBS), homogenized, and suspended in PBS. The suspension was centrifuged at 1600 3*g* for 10 min. The supernatant was collected, ethanol was added to remove proteins, and the mixture was heated at 90 °C to evaporate the ethanol. The protein-free samples were used to evaluate the MIC in brain tissue by HPLC and bioassay.

5.11.2. Radical scavenging assays

5.11.2.1. DPPH radical scavenging assay. The antioxidant activity of the ten compounds **2**, **6**, **9**, **10**, **12**–**14**, **16**, **21a**,**b** and the standard were assessed on the basis of the radical scavenging effect of the stable DPPH$^\bullet$ free radical [30]. Weighed quantities of the ten compounds were dissolved in distilled DMSO and used. Compound **21a** was not fully soluble in DMSO (even after treating its solution for 5 min in an ultrasonic bath). Solution of ascorbic acid used as standard for this study was prepared in distilled H_2O. All these solutions were serially diluted with respective solvents to get lower dilutions.

10 μl of each compound or standard (from 0.0 μM/ml to 100 μM/ml) was added to 90 μl of DPPH$^\bullet$ in methanol solution (100 μM) in a 96 well microtitre plate. After incubation in the dark at 37 °C for 30 min, the decrease in absorbance of each solution was measured at 515 nm using ELISA micro plate reader (Bio Rad Laboratories Inc., California, U.S.A., Model 550). Absorbance of blank sample containing the same amount of DMSO and DPPH$^\bullet$ solution was prepared and measured as well. The experiment was carried out in triplicate. The scavenging potential was compared with a solvent control (0% radical scavenging) and ascorbic acid. Radical scavenging activity was calculated by the following formula:

$$\%\text{Reduction of absorbance} = [(A_B - A_A)/A_B] \times 100$$

where: A_B – absorbance of blank sample; A_A – absorbance of tested extract solution ($t = 30$ min).

5.11.3. Reaction with reactive nitrogen species

5.11.3.1. Synthesis of peroxynitrite. Peroxynitrite ($ONOO^-$) was synthesized as previously described [26]. Briefly, an acidic solution (0.6 M HCl) of H_2O_2 (0.7 M) was mixed with KNO_2 (0.6 M) on ice for 1 s and the reaction quenched with ice-cold NaOH (1.2 M). Residual H_2O_2 was removed by mixing with granular MnO_2 prewashed with NaOH (1.2 M). The stock solution was filtered and then frozen overnight (−20 °C) and the top layer of the solution collected for the experiment. Concentrations of stock $ONOO^-$ were redetermined before each experiment at 302 nm using a molar absorption coefficient of 1670 $cm^{-1}\,M^{-1}$. Concentrations of 200–250 mM were usually obtained. Once thawed, $ONOO^-$ solutions were kept in ice for no longer than 30 min before use.

5.11.3.2. Reaction of compounds with peroxynitrite. The ability of tested compounds to inhibit peroxynitrite-induced tyrosine nitration was investigated *via* reaction of equimolar concentrations (100 μM) of tyrosine and peroxynitrite in the presence of increasing concentrations of each tested compound (0–300 μM) in 100 mM phosphate buffer, pH 7.4 at 37 °C for 15 min. Snap-freezing reaction mixtures prior to HPLC analysis successfully terminated reactions. The formation of 3-nitrotyrosine (3-NT) was monitored by HPLC analyzed with photodiode array detection (see below). 3-NT formed was characterised and quantified by use of an authentic standard (elution time and unique spectral characteristics).

5.11.3.3. HPLC analysis. Reaction mixtures were analyzed using reverse-phase HPLC. Analysis was performed on an Agilent 1100 system with a Zorbax ODS C18 column (150 × 4.6 mm i.d., 4 μm) and guard column (15 × 4.6 mm i.d., 4 μm). Mobile phase A consisted of methanol/water/5 N HCl (5/94.9/0.1 v/v/v) and mobile phase B of acetonitrile/water/5 N HCl (50/49.9/0.1 v/v/v). The following gradient system was used (min/% acetonitrile): 0/0, 5/0, 40/50, 60/100, 65/100, and 65.1/0 with a flow rate of 0.7 ml/min. The eluent was monitored by photodiode array detection at 280 nm for 3-NT measurements with spectra of products obtained over the 220–600 nm range.

Appendix. Supplementary data

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.ejmech.2009.10.033.

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