Postprint of: Olszewska T., Gajewska E. P., Milewska M. J., Alternative method for the synthesis of imidazo[5,1-f][1,2,4]triazin-4(3H)-one—a substrate for the preparation of phosphodiesterase (5) inhibitors, Tetrahedron, Vol. 69, Iss. 2 (2013), pp. 474-480, DOI: 10.1016/j.tet.2012.11.037

Alternative method of synthesis of imidazo[5,1-*f*][1,2,4]triazin-4(3*H*)-one – a substrate for preparation of phosphodiesterase(5) inhibitors

Teresa Olszewska, Ewa P. Gajewska and Maria J. Milewska*

Department of Organic Chemistry, Gdańsk University of Technology, 80-233 Gdańsk, Poland;

ARTICLE INFO

Article history:
Received
Received in revised form
Accepted
Available online

Keywords:
heterocycle
synthesis of imidazotriazinones
Vardenafil
cGMP-PDE5 inhibitors

ABSTRACT

Imidazo[5,1-*f*][1,2,4]triazin-4(3*H*)-ones, as isosteres of purine, are of interest for pharmaceutical research as potential substrates for the synthesis of cGMP-PDE5 inhibitors. We present a novel, alternative method for the synthesis of imidazotriazinones, that differs from the previously reported ones with respect to the way of constructing a triazinone ring in the molecule. The key step in our approach is condensation of an appropriate α-keto-ester with amidrazones, leading to the triazinone heterocycle. Several different substituted imidazolotriazinones have been synthesized in this manner.

1. Introduction

Phosphodiesterases (PDEs) are a large family of enzymes which are responsible for breaking the phosphodiester bonds in biological molecules and some of them are involved in regulation of physiological functions.[1,2] Some of the PDEs are drug targets for the treatment of various diseases, including: heart failure, depression, asthma, inflammation and erectile disfunction.[3-5] In particular, phosphodiesterase 5 (PDE5), which is involved in hydrolysis of a secondary messenger, cyclic guanosine monophosphate (cGMP), present in the corpus cavernosum tissue, plays an important role in mediating the sexual response.[6-8] Inhibition of PDE5 increases the cGMP level, triggering erection *via* relaxation of the penile arterioles.[9] Selective inhibitors of PDE5 have a great clinical significance in treatments of the erectile dysfunction disease and their other therapeutic applications are being proposed and investigated.[10-11] There are three commercially available drugs acting as PDE5 inhibitors, namely: sildenafil citrate (the active indegredient in Viagra), vardenafil (Levitra) and tadalafil.[12] A core structure in vardenafil **2**, which has been approved by the FDA and launched in 2003, is imidazo[5,1-*f*][1,2,4]triazin-4(3*H*)-one – general structure **1** (Fig. 1).

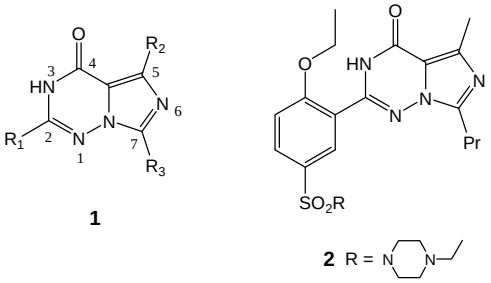
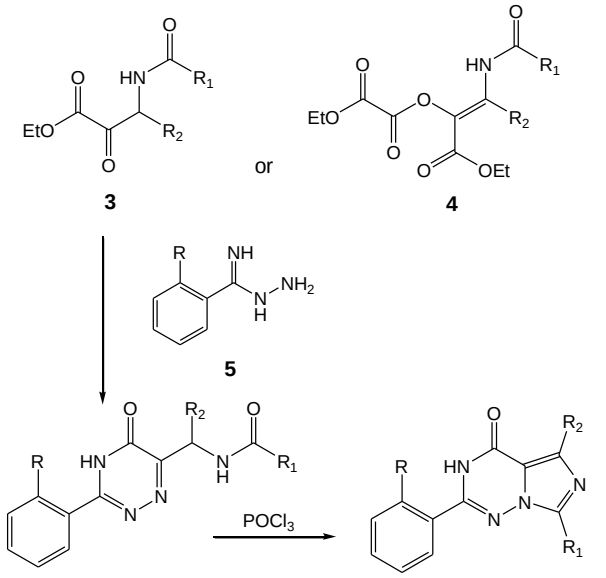


Figure 1. Chemical structure of imidazo[5,1-*f*][1,2,4]triazin-4(3*H*)-one **1** and vardenafil **2**

Several method for synthesis of imidazotriazinones have been reported so far.[13-17] They can be divided into two major groups depending on the sequence of steps of ring construction. These in which the triazinone ring is built at the beginning are generally based on the method described by Charles *et al.*,[17] where the ring is formed by condensation of an acyloamino-α-keto-ester **3** or enol ester **4** with an benzamidrazone **5** or generally amidrazone, as shown in Scheme 1.

* Corresponding author. Fax +48 58 347 26 94; tel. +48 58 347 11 34; E-mail address: marmilew@pg.gda.pl



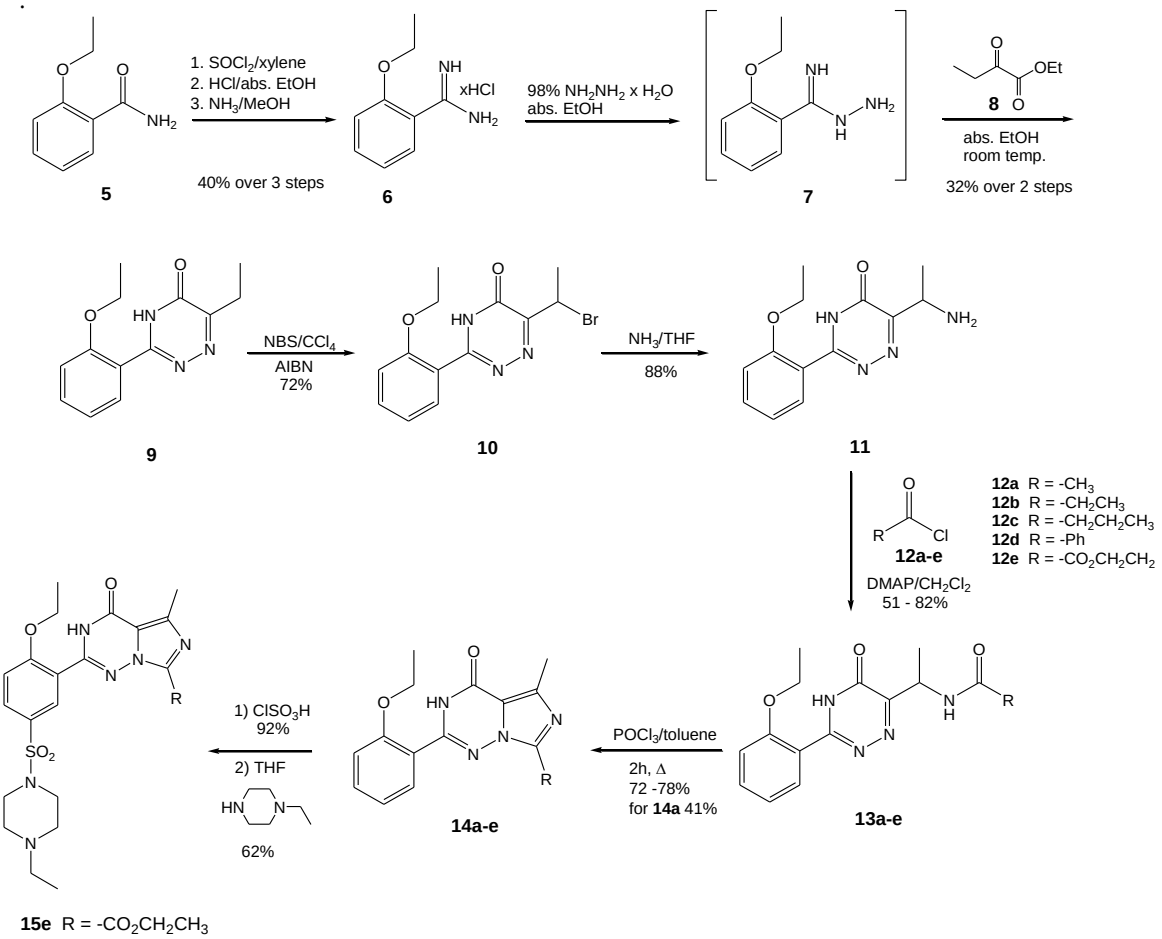
Scheme 1. Synthesis of imidazo[5,1-*f*][1,2,4]triazin-4(*3H*)-ones[17]

The main drawback in this approach is availability of the active intermediate **3** or **4**, both of which are obtained from α-amino acids and ethyl oxalate *via* the Dakin-West reaction.[18,19] It is well known that these compounds are very capricious and cannot be obtained with purity greater than 50%. This limitation led us to investigate a novel route for the construction of the imidazo[5,1-*f*][1,2,4]triazin-4(3*H*)-one core which does not require the reactive intermediate **3** or **4** and gives rise to the possibility of synthesis of different substituted imidazotriazinones.

2. Results and discussion

In the proposed strategy, a triazinone ring is formed by condensation of benzamidrazone **7** with the stable 2-oxo-butyric acid ethyl ester **8**. The sequence of reactions leading to the eventual formation of the imidazotriazinone is shown in Scheme 2.

The substituted benzamidrazone **7**, an essential intermediate for the construction of the triazinone ring, was prepared in four steps. The commercially available 2-ethoxybenzamide **4** was used as a starting substrate which upon treatment with thionyl chloride in xylene was dehydrated to the 2-ethoxybenzonitrile with 90% yield. This intermediate was then converted to the benzamidine hydrochloride by subsequent reactions with gaseous dry hydrogen chloride and ammonia in absolute alcohols. Alternatively, compound **7** can be obtained *via* reaction between 2-ethoxybenzonitrile and $AlMeClNH_2$,[20] prepared from $AlMe_3$ and NH_4Cl in one step or in reaction with lithium hexamethyldisilazane (LHMDS) in THF at room temperature, as described by Mao and co-workers.[21] Hydrazinolysis of benzamidine hydrochloride **6** to the intermediate **7** was performed with hydrazine hydrate in ethanol at 0-5°C. Benzamidrazone **7** is an unstable compound but it can be isolated as a picrate salt with 56% yield. The key step of the whole synthesis is condensation of benzamidrazone **7** with 2-oxo-butyric acid ethyl ester **8**, leading to the formation of a triazinone ring in compound **9**. Due to the high reactivity of benzamidrazone **7**, several products may be formed at this stage. The highest yield of expected **7** was achieved when a freshly prepared benzamidrazone was used without purification. Anhydrous ethanol was found to be the optimal solvent in this reaction; the yield of condensation was poorer when the reaction was run in methanol. The imidazole ring was then built up on the imidazotriazinone molecule *via* bromination at the benzylic position, followed by amination with ammonia and a final dehydration-cyclisation in the presence of $POCl_3$.



Scheme 2.

Bromination of the benzylic position in the compound **9** was performed using NBS as a source of bromine. Treatment of the resulting intermediate **10** with 14% ammonia in THF afforded **11** in an unequivocal way. When MeOH was used as a solvent in this reaction, a methyloxy derivative was formed together with **11**. The amine derivative **11** was treated with acid chlorides **12a-e** in dichloromethane at 0 °C, in the presence of a catalytic amount of DMPA. The reaction afforded amides **13a-e** in a high yields, 80-90%. Cyclisation of compounds **13a-e** with phosphoryl chloride, either in methylene chloride or in toluene, gave final products, compounds **14a-e**, containing the required imidazo[5,1-*f*][1,2,4]triazin-4(3*H*)-one core (compound **14c** is identical with the vardenafil core), of high purity and with overall good yield. Structure of all final products was confirmed by MS and NMR analysis. One of them, namely **14b** was crystallized as large crystals and its 3D structure was determined by X-ray diffraction. The structure is shown in Fig. 2.

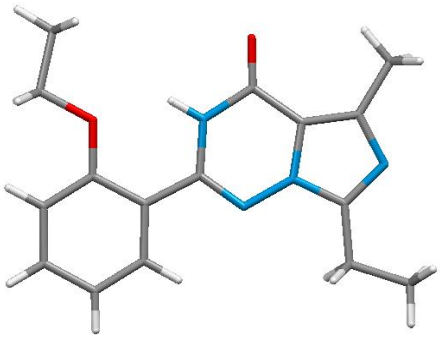
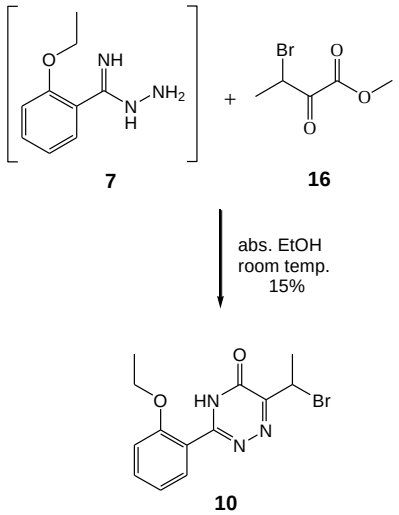


Figure 2. X-ray structure of **14b**.

Because our goal was to access imidazo[5,1-*f*][1,2,4]triazin-4(3*H*)-one core from stable and easily accessible substrates, apart from using 2-oxo-butyric acid ethyl ester **8** as a starting material in our synthetic route (Scheme 2), we also tested the possibility of getting compound **10** by condensation of benzamidrazone **7** with 3-bromo-2-oxo-butyric acid ethyl ester **16** which was obtained in the reaction of ethyl 2-oxobutanoate with $CuBr_2$ in yields as high as 87% after purification.



Scheme 3.

The cyclization carried out in ethanol led to the required product **10**. However, presumably due to the higher reactivity of 3-bromo-2-oxo-butyric acid ethyl ester **16** comparing to 2-oxo-butyric acid ethyl ester **8,** the yield was moderately low.

The synthesized compounds **14a-d** can be easily transformed to vardenafil and its analogues by two subsequent reaction, namely chlorosulphonation and sulphonamide formation, conditions of which are described in literature.[22] Only derivative **14e**, which is to our knowledge a new compound, has not been transformed in this way. Even if this transformation was not aim of our work, we decided to check whether the reaction condition could affect the substituent ester function at the 7-position of imidazotriazinone **14e**. As shown in Scheme 2, chlorosulphonation of the compound **14e** in chlorosufonic acid at 0 °C and following after amination with *N*-ethylpiperazine proceeded smoothly and selectively at the 5'-position of the phenyl ring to afford the target product **15e**. As a new analogue of vardenafil, compound **15e** is worth testing for its pharmacological properties.

3. Conclusion

In conclusion, we have developed a new, alternative method for the synthesis of imidazotriazinones – substrates for the synthesis of potential PDE5 inhibitors. The main advantage of the novel strategy is the use of the more stable substrate in the key condensation, which results in an excellent repeatability of the reaction at this stage and higher yields. Following our procedure, five differently substituted imidazotriazinones were synthesized and this synthesis demonstrates the potential of the novel method for preparation of a wide range of compounds based on the imidazo[5,1-*f*][1,2,4]triazin-4(3*H*)-ones ring system. The method was applied for a formal synthesis of vardenafil substrate and could be alternative for scale-up preparation of vardenafil.

4. Experimental

4.1. General

All solvents and reagents were used as obtained from commercial source. ^{1}H NMR and ^{13}C NMR spectra were obtained at 500 and 125 MHz (Varian Unity Plus), respectively, and the deuteriated solvents were used as internal lock. Infrared spectra were recorded on a FT-IR Bruker IFS 66 instrument. Band positions are reported in reciprocal centimeters (cm^{-1}). Melting points were determined on a melting point apparatus equipped with thermometer and were uncorrected. Column chromatography was carried out in silica gel 0.040-0.063 mm. The mass spectra analyses were carried out using the MALDI-TOF Bruker BiFlex III mass spectrometer. Elemental analyses were recorded on a Perkin Elmer 240C Elemental Analyzer.The reason is that the Abstract should be understandable in itself to be suitable for storage in textual information retrieval systems.

*4.2. 2-ethoxybenzamidine hydrochloride **6***

The 2-ethoxybenzonitrile was prepared from 2-ethoxybenzamide **5** according to the method reported by Nowakowski[23] in 89 % yield (lit.[23] yield 92 %); R_f (hexane/EtOAc 9:1) 0.45. ^{1}H NMR ($CDCl_3$, δ ppm): 7.57-7.51 (m, 2H, 4-H and 5-H of C_6H_4), 7.01-7.00 (d, J = 7.3 Hz, 1H, 6-H of C_6H_4), 6.98-6.96 (d, J=8.8 Hz, 1H, 3-H of C_6H_4), 4.19-4.15 (q, J = 7 Hz, 2H, OCH_2), 1.51-1.48 (t, J = 7.1 Hz, 3H, CH_3). IR (crystal) ν: 2231 cm^{-1} (C≡N). Ethyl 2-ethoxybenzimidate hydrochloride was synthesized by passing dry $HCl_{(gas)}$ through the solution of 2-ethoxybenzonitrile in anhydrous ethyl alcohol. This compound was obtained as a white solid; yield 46 %, mp 49-54 °C; R_f (hexane/EtOAc 7:3) 0.36. ^{1}H NMR ($CDCl_3$, δ ppm): 13.01 (bs,

1H, NH), 10.14 (bs, 1H, NH), 8.05-8.03 (dd, *J* = 1.7 and 8 Hz, 1H, 6-H of C_6H_4), 7.74-7.70 (m, 1H, 4-H of C_6H_4), 7.18-7.14 (m, 2H, 3-H and 5-H of C_6H_4), 5.06-5.01 (q, *J* = 7 Hz, 2H, $NCOCH_2$), 4.41-4.37 (q, *J* = 7 Hz, 2H, $OCOCH_2$), 1.64-1.61 (t, *J* = 7.1 Hz, 3H, $NCOCH_2CH_3$), 1,59-1,56 (t, *J* = 6.8 Hz, 3H, $OCOCH_2CH_3$). 2-Ethoxybenzamidine hydrochloride **6** was prepared from ethyl 2-ethoxybenzimidate hydrochloride and NH_3/methanol. Crude product was purified by crystallization (methanol/ethyl ether) to provide **6** as a white solid crystal (96 % yield), mp 190-192 °C; (lit.[24] yield 91%, mp 195-196 °C); R_f (n-butanol/acetic acid/water 4:4:1) 0.31. ^{1}H NMR ($CDCl_3$+DMSO-d_6, δ ppm): 9.76 (bs, 2H, NH), 8.41 (bs, 2H, NH), 7.81-7.80 (d, *J* = 7.8 Hz, 1H, 6-H of C_6H_4), 7.55-7.51 (m, 1H, 4-H of C_6H_4), 7.07-7.04 (t, *J* = 7.6 Hz, 1H, 5-H of C_6H_4), 7.01-6.99 (d, *J* = 8.3 Hz, 1H, 3-H of C_6H_4), 6.69 (bs, 2H, NH_2), 4.17-4.13 (q, *J* = 7 Hz, 2H, OCH_2), 1.45-1.42 (t, *J* = 7.1 Hz, 3H, CH_3). IR (crystal) ν: 1662 cm^{-1} (C=N). MS-ESI (matrix DHB): *m/z* $[M+H]^+$ calcd for $C_9H_{12}N_2O$: 165.095; found 165.1.

*4.3. ethyl 2-oxobutanoate **8***

The ethyl 2-oxobutanoate **8** was prepared from 2-ketobutyric acid 25 g (0.245 mol), *p*-toluenesulfonic acid 0.5 g, absolute ethanol 140 ml and toluene 80 ml using a Dean and Stark apparatus. The reaction proceeded for 48 h at reflux. After the standard work-up purification of the product was performed by distillation under reduced pressure (water pump). The ethyl 2-oxobutanoate **8** was collected as a fraction boiling at 70-75 °C (20 mm Hg), yield 65 % (lit.[25] bp_{18} 65 °C). ^{1}H NMR ($CDCl_3$, δ ppm): 4.35-4.31 (q, *J* = 7.2 Hz, 2H, $OCOCH_2$), 2.90-2.86 (q, *J* = 7.2 Hz, 2H, $OC\text{-}CH_2$), 1.39-1.36 (t, *J* = 7.1 Hz, 3H, $OCOCH_2CH_3$), 1.16-1.13 (t, *J* = 7.3 Hz, 3H, $OC\text{-}CH_2CH_3$).

*4.4. 3-(2-ethoxyphenyl)-6-ethyl-4H-[1,2,4]triazin-5-one **9***

To the ice-cold, stirred suspension of amidine hydrochloride **6** 2.1 g (0.01 mol) in absolute ethanol (10 mL), the 98% solution of hydrazine hydrate (0.51 mL, 0.01 mol) was added. The mixture was allowed to stand at 0 to 5 ºC overnight and then the precipitated ammonium chloride was filtered off. Ethyl 2-oxobutanoate **8** 1.3 g (0.01 mol) dissolved in anhydrous methanol (10 mL) was added to the filtrate, resulting in an immediate formation of a white solid. The resulting mixture was allowed to stand at room temperature for 8 hours. The solid (inorganic salt) was then filtered off and the filtrate was concentrated under reduced pressure. Dissolution of the residue in chloroform (10 mL) led to the precipitation of another portion of inorganic salt which was filtered off. The residue was concentrated and purified by chromatography on a silica gel column eluted with 30-50% (v/v) ethyl acetate in petroleum ether to give the triazine **9** as a pale yellow oil. This oil was crystallised from ethyl acetate/hexane to give the product as a white solid 0.85 g (32 %); mp 114-116 °C; R_f (hexane/EtOAc, 1:1) 0.28. ^{1}H NMR ($CDCl_3$, δ ppm): 12.10 (brs, 1H, NH···O), 8.58-8.56 (dd, *J* = 1.7 and 8.1 Hz, 1H, 6-H of C_6H_4), 7.55-7.52 (t, *J* = 8.8 Hz, 1H, 4-H of C_6H_4), 7.15-7.12 (t, *J* = 7.6 Hz, 1H, 5-H of C_6H_4), 7.06-7.05 (d, *J* = 8.3 Hz, 1H, 3-H of C_6H_4), 4.36-4.32 (q, *J* = 7 Hz, 2H, $OCOCH_2$), 2.83-2.78 (q, *J* = 7.5 Hz, 2H, $C\text{-}CH_2$), 1.62-1.59 (t, *J* = 7.1 Hz, 3H, CH_3CH_2O), 1.28-1.25 (t, *J* = 7.3 Hz, 3H, CH_3CH_2C). ^{13}C NMR ($CDCl_3$, δ ppm): 160.7, 158.2, 158.1, 135.3, 132.2, 122.4, 116.6, 112.8, 65.7, 19.3, 15.0, 9.3. Anal. Calcd for $C_{13}H_{15}N_3O_2$: C, 63.66; H, 6.16; N, 17.13. Found: C, 63.55; H, 6.19; N, 17.19.

*4.5. 6-(1-bromoethyl)-3-(2-ethoxyphenyl)-4H-[1,2,4]triazin-5-one **10***

To the magnetically stirred solution of 3-(2-ethoxyphenyl)-6-ethyl-4*H*-[1,2,4]triazin-5-one (**9**) 3.36 g (0.0137 mol) in anhydrous carbon tetrachloride (350 mL) under an argon atmosphere, *N*-bromosuccinimide 2.7 g (0.015 mol) and 2,2'-azo-bis-isobutyrylnitrile (174 mg) were added. The reaction mixture was stirred under reflux for 14 h and then cooled to room temperature. The solvent was evaporated *in vacuo* and the residue was treated with chloroform (200 mL) and water (150 mL). The mixture was shaken vigorously until complete dissolution of all solid. The chloroform layer was separated, washed with water, dried over anhydrous magnesium sulphate and filtered. The solvent was evaporated. The crude product was purified by column chromatography on silica gel eluted with hexane: ethyl acetate 1:1 (v/v). The final product **10** was crystallised from ethyl acetate as a light yellow solid: 3.19 g (yield 72%); mp 116-121 °C; R_f (hexane/EtOAc, 1:1) 0.5. ^{1}H NMR ($CDCl_3$, δ ppm): 8.57-8.56 (d, *J* = 8.3 Hz, 1H, 6-H of C_6H_4), 7.57-7.54 (t, *J* = 7.8 Hz, 1H, 4-H of C_6H_4), 7.16-7.13 (t, *J* = 7.6 Hz, 1H, 5-H of C_6H_4), 7.07-7.06 (d, *J* = 8.3 Hz, 1H, 3-H of C_6H_4), 5.49-5.45 (q, *J* = 6.8 Hz, 2H, CH_2Br), 4.38-4.33 (q, *J* = 7 Hz, 2H, CH_2O), 2.01 (d, *J* = 6.8 Hz, 3H, CH_3CH), 1.63-1.60 (t, *J* = 6.8 Hz, 3H, CH_3CH_2). ^{13}C NMR ($CDCl_3$, δ ppm): 162.2, 158.1, 157.9, 154.6, 135.2, 132.1, 122.2, 116.6, 112.8, 65.7, 44.7, 17.1, 15.0. Anal. Calcd for $C_{13}H_{14}BrN_3O_2$: C, 48.17; H, 4.35; N, 12.96. Found: C, 48.31; H, 4.38; N, 12.91.

*4.6. General procedure for the synthesis of the N-{1-[3-(2-ethoxyphenyl)-5-oxo-4,5-dihydro[1,2,4]triazin-6-yl]ethyl}-alkanoamide **13***

The bromoderivative **10** (0.6 g, 0.00185 mol) placed in a round-bottomed flask was treated with NH_3/THF solution (60 mL). The reaction flask was closed tightly and allowed to stand at room temperature with occasional shaking until all substrate **10** was consumed (TLC control). The solvent was removed by evaporation and the oily residue was dissolved in 30 mL of dichloromethane, washed twice with water (10 mL), dried over anhydrous $MgSO_4$ and evaporated to dryness under reduced pressure. The crude amino product **11** 0.42 g (yield 88%) was used without further purification in the next step. To the stirred, ice-cooled solution of the crude amino compound **11** 0.43 g (0.00165 mol) in dry dichloromethane (20 mL), protected from moisture by a calcium chloride drying tube, triethylamine 0.46 mL (0,0033 mol) was added. The mixture was stirred for 5 minutes and the acyl chloride **12a-e** (0.0018 mol) was added dropwise. After 10 minutes the cooling bath was removed and the reaction mixture was allowed to stand for 1 hour at room temperature. The reaction was quenched by the addition of water (10 mL). The organic layer was washed with water, brine, and dried over anhydrous $MgSO_4$. The solvent was removed *in vacuo* and the crude product **13** was purified by two consecutive silica gel column chromatography separations. In the first one, an ethyl acetate/petroleum ether mixture was used as a eluting solvent and the second column was developed with the ethyl acetate/methanol mixture 6:0.5 (v/v).

4.6.1 ***N*-{1-[3-(2-ethoxy-phenyl)-5-oxo-4,5-dihydro-[1,2,4]triazin-6-yl]ethyl}acetamide 13a**. The product **13a** was obtained as a light yellow oil; yield 0.29 g (51 %); R_f (EtOAc/EtOH, 24:0.5) 0.25. ^{1}H NMR ($CDCl_3$, δ ppm): 12.12 (brs, 1H, NH···O), 8.57 (d, *J* = 7.8 Hz, 1H, 6-H of C_6H_4), 7.58-7.54 (t, *J* = 7.8 Hz, 1H, 4-H of C_6H_4), 7.18 (t, *J* = 7.3 Hz, 1H, 5-H of C_6H_4), 7.08 (d, *J* = 8.3 Hz, 1H, 3-H of C_6H_4), 6.89 (brd, *J* = 8.8 Hz, 1H, NH), 5.25-5.22 (m, 1H, CHN), 4.37-4.33 (q, *J* = 6.8 Hz, 2H, CH_2O), 2.0 (s, 3H, CH_3CO), 1.62-1.60 (t, *J* = 7.1 Hz, 3H, CH_3CH_2O), 1.55-1,53 (d, *J* = 7.3 Hz, 3H, CH_3CH). ^{13}C NMR ($CDCl_3$, δ ppm): 169.7, 158.2, 158.1, 135.3, 132.2, 122.4, 116.5, 112.9, 65.7, 47.9, 23.7, 19.3, 15.0. Anal. Calcd for $C_{15}H_{18}N_4O_3$: C, 59.59; H, 6.0; N, 18.53. Found: C, 59.65; H, 6.02; N, 18.47.

4.6.2 ***N*-{1-[3-(2-ethoxy-phenyl)-5-oxo-4,5-dihydro-[1,2,4]triazin-6-yl]ethyl}propioamide 13b**. The product **13b** was obtained as a light yellow solid, yield 0.39 g (68 %), mp. 72 –75 ºC; R_f (EtOAc/EtOH, 24:0.5) 0.27. ^{1}H NMR ($CDCl_3$, δ ppm): 8.6-8.59 (d, *J* = 7.8 Hz, 1H, 6-H of C_6H_4), 7.61-7.58 (t, *J* = 7.8 Hz, 1H, 4-H of

C_6H_4), 7.2-7.17 (t, *J* = 7.6 Hz, 1H, 5-H of C_6H_4), 7.1-7.08 (d, *J* = 8.3 Hz, 1H, 3-H of C_6H_4), 6.98-6.96 (d, *J* = 7.3 Hz, 1H, NHCO), 5.31-5.28 (qv, *J* = 7.3 Hz, 1H, CHN), 4.39-4.35 (q, *J* = 6.8 Hz, 2H, CH_2O), 2.29-2.24 (q, *J* = 7.6 Hz, 2H, CH_2CO), 1.64-1.61 (t, *J* = 6.8 Hz, 3H, C**H**$_3$$CH_2O$), 1.57-1.55 (d, *J* = 6.8 Hz, 3H, C**H**$_3$CH), 1.19-1.16 (t, *J* = 7.3 Hz, 3H, C**H**$_3$$CH_2C$). ^{13}C NMR ($CDCl_3$, δ ppm): 169.7, 158.2, 158.1, 135.3, 132.2, 122.4, 116.5, 112.9, 65.7, 47.9, 23.7, 19.3, 15.0, 14.8. Anal. Calcd for $C_{16}H_{20}N_4O_3$: C, 60.75; H, 6.37; N, 17.71. Found: C, 60.59; H, 6.39; N, 17.79.

4.6.3. ***N*-{1-[3-(2-ethoxyphenyl)-5-oxo-4,5-dihydro-[1,2,4]triazin-6-yl]ethyl}butyroamide 13c**. The product **13c** was obtained as a light yellow oil yield 0.42 g (69 %). ^{1}H NMR ($CDCl_3$, δ ppm): 12.42 (s, 1H, NH···O), 8.57-8.55 (dd, *J* = 1.5 and 7.8 Hz, 1H, 6-H of C_6H_4), 7.58-7.55 (td, *J* = 8.5 and 1.5 Hz, 1H, 4-H of C_6H_4), 7.17-7.14 (t, *J* = 7.8 Hz, 1H, 5-H of C_6H_4), 7.08-7.06 (d, *J* = 8.3 Hz, 1H, 3-H of C_6H_4), 6.96-6.94 (d, *J* = 8.8 Hz, 1H, NHCO), 5.26-5.23 (dd, *J* = 9 and 6.8 Hz, 1H, CHN), 4.37-4.33 (q, *J* = 6.8 Hz, 2H, CH_2O), 2.2-2.17 (t, *J* = 7.6 Hz, 2H, CH_2CO), 1.68-1.63 (m, 2H, CH_3C**H**$_2$$CH_2$), 1.62-1.59 (t, *J* = 6.8 Hz, 3H, C**H**$_3$$CH_2O$),1.52 (d, *J* = 7.3 Hz, 3H, C**H**$_3$CH), 0.94-0.91 (t, *J* = 7.3 Hz, 3H, C**H**$_3$$CH_2$). ^{13}C NMR ($CDCl_3$, δ ppm): 172.7, 158.0, 135.2, 132.0, 122.3, 116.5, 112.9, 65.7, 47.4, 39.0, 29.9, 19.5, 19.3, 15.0, 14.0. Anal. Calcd for $C_{17}H_{22}N_4O_3$: C, 61.80; H, 6.71; N, 16.96. Found: C, 61.67; H, 6.68; N, 16.92.

4.6.4. ***N*-{1-[3-(2-ethoxy-phenyl)-5-oxo-4,5-dihydro-[1,2,4]triazin-6-yl]ethyl}benzamide 13d**. The product **13d** was obtained as a light yellow oil yield 0.55 g (82 %). ^{1}H NMR ($CDCl_3$, δ ppm): 12.80-12.20 (brs, 1H, NH···O), 8.57-8.55 (dd, *J* = 1.7 and 8.1 Hz, 1H, 6-H of C_6H_4), 7.84-7.83 (d, *J* = 7.3 Hz, 3H, C_6H_5), 7.58-7.54 (td, *J* = 1.7 and 8.8 Hz, 1H, 4-H of C_6H_4), 7.50-7.47 (t, *J* = 7.3 Hz, 1H, C_6H_5), 7.43-7.40 (t, *J* = 7.3 Hz, 2H, C_6H_5), 7.17-7.14 (t, *J* = 7.6 1H, 5-H of C_6H_4), 7.07 (d, *J* = 8.3 Hz, 1H, 3-H of C_6H_4), 5.50-5.47 (m, 1H, CHN), 4.36-4.32 (q, *J* = 7 Hz, 2H, CH_2O), 1.66-1.64 (d, *J* = 6.8 Hz, 3H, C**H**$_3$CH), 1.61-1.59 (t, *J* = 6.8 Hz, 3H, C**H**$_3$$CH_2O$). ^{13}C NMR ($CDCl_3$, δ ppm): 166.8, 158.1, 135.3, 134.5, 132.1, 131.8, 128.7, 127.4, 122.3, 116.5, 112.9, 65.7, 48.2, 19.7, 15.0. Anal. Calcd for $C_{20}H_{20}N_4O_3$: C, 65.92; H, 5.53; N, 15.38. Found: C, 65.85; H, 5.51; N, 15.32.

4.6.5. ***N*-{1-[3-(2-ethoxyphenyl)-5-oxo-4,5-dihydro-[1,2,4]triazin-6-yl]ethyl}oxalamic acid ethyl ester 13e**. The product **13e** was obtained as a light yellow oil yield 0.38 g (57 %); R_f (hexane/EtOAc, 3:7) 0.29. ^{1}H NMR ($CDCl_3$, δ ppm): 12.55-11.50 (brs, 1H, NH···O), 8.57-8.55 (dd, *J* = 1.5 and 7.8 Hz, 1H, 6-H of C_6H_4), 8.30-8.29 (d, *J* = 8.8 Hz, 1H, NHCO), 7.58-7.55 (td, *J* = 8.5 and 1.5 Hz, 1H, 4-H of C_6H_4), 7.17-7.13 (t, *J* = 7.8 Hz, 1H, 5-H of C_6H_4), 7.08-7.06 (d, *J* = 8.3 Hz, 1H, 3-H of C_6H_4), 5.33-5.30 (m, 1H, CHN), 4.38-4.33 (m, 4H, CH_2O), 1.63-1.59 (m, 6H, C**H**$_3$CH+C**H**$_3$$CH_2O$), 1.39-1.36 (t, *J* = 7.1 Hz, 3H, C**H**$_3$$CH_2O$). ^{13}C NMR ($CDCl_3$, δ ppm): 160.6, 158.1, 156.1, 135.3, 132.1, 122.3, 116.4, 112.9, 65.8, 63.4, 47.4, 19.2, 15.0. Anal. Calcd for $C_{18}H_{22}N_4O_5$: C, 57.75; H, 5.92; N, 14.96. Found: C, 57.63; H, 5.93; N, 14.91.

*4.7. General procedure for the synthesis of the 2(2-ethoxy-phenyl)-7-alkyl-5-methyl-imidazo[5,1-f][1,2,4]triazin-4(3H)-one **14***

To the magnetically stirred solution of **13** (0.0010 mol) in toluene (35 mL), phosphorus oxychloride (0.17 ml, 0.0019 mol) was added at room temperature. The resulting mixture was heated under reflux for 2 h and then cooled to room temperature. The solvent and an excess of phosphorus oxychloride were evaporated *in vacuo* and the residue was treated with saturated aqueous sodium bicarbonate solution (15 mL) and chloroform (15 mL). The mixture was shaken vigorously until all solid had dissolved. The chloroform layer was separated and the aqueous phase was extracted with another portion of chloroform (2×15 mL). The chloroform extracts were combined, dried over anhydrous magnesium sulphate, filtered and the solvent was evaporated. The residue was purified by column chromatography on silica gel (eluted with chloroform) afforded a product which was then crystallised from an appropriate solvent.

4.7.1. **2-(2-ethoxyphenyl)-5,7-dimethyl-imidazo[5,1-*f*][1,2,4]-triazin 4(*3H*)-one 14a**. The title compound was prepared from 0.12 g (0.0004 mol) of **13a.** The final product was crystallised from ethyl acetate/ethyl ether to give 46 mg of **14a** (41%) as white crystals, mp 209-210 ºC; R_f (EtOAc/EtOH, 24:0.5) 0.52. ^{1}H NMR ($CDCl_3$, δ ppm): 10.00 (brs, 1H, NH···O), 8.19-8.17 (dd, *J* = 1,5 and 7,8 Hz, 1H, 6-H of C_6H_4), 7.51-7.48 (t, *J* = 8.5 Hz, 1H, 4-H of C_6H_4), 7.13-7.10 (t, *J* = 7.6 Hz, 1H,5-H of C_6H_4), 7.05-7.04 (d, *J* = 8.3 Hz, 1H, 3-H of C_6H_4), 4.28-4.24 (q, *J* = 7 Hz, 2H, CH_2O), 2.65 (s, 3H, CH_3C), 2.63 (s, 3H, CH_3C), 1.58-1.55 (t, *J* = 7.1 Hz, 3H, CH_3). MS-ESI (matrix CCA): *m/z* $[M+H]^+$ calcd for $C_{15}H_{16}N_4O_2$: 285.1273; found 285.3. Anal. Calcd for $C_{15}H_{16}N_4O_2$ (284): C, 63.37; H, 5.67; N, 19.71. Found: C, 63.61; H, 5.65; N, 19.65.

4.7.2. **2-(2-ethoxyphenyl)-5-methyl-7-ethyl-imidazo[5,1-*f*][1,2,4]-triazin-4(*3H*)-one 14b**. The title compound was prepared from 1.028 g (0.0032 mol) of **13b**. The final product was crystallised from ethyl acetate to afford 0.7 g of **14b** (72 %) as a light yellow solid, mp 176 -178 ºC; R_f (EtOAc/EtOH, 24:0.5) 0.64. ^{1}H NMR ($CDCl_3$, δ ppm): 10.03 (bs, 1H, NH···O), 8.20-8.18 (dd, *J* = 1.5 and 7.8 Hz, 1H, 6-H of C_6H_4), 7.54-7.51 (dt, *J* = 1.7 and 7.8 Hz, 1H, 4-H of C_6H_4), 7.16-7.13 (dt, *J* = 0.9 and 8.3 Hz, 1H, 5-H of C_6H_4), 7.08-7.06 (d, *J* = 8.3 Hz, 1H, 3-H of C_6H_4), 4.31-4.27 (q, *J* = 7 Hz, 2H, CH_2O), 3.12-3.07 (q, *J* = 7.65 Hz, 2H, CH_2C), 2.68 (s, 3H, CH_3C), 1.6-1.57 (t, *J* = 7.1 Hz, 3H, C**H**$_3$$CH_2O$), 1.46-1.43(t, *J* = 7.6 Hz, 3H, C**H**$_3$$CH_2C$). MS-ESI (matrix CCA): *m/z* $[M+H]^+$ calcd for $C_{16}H_{18}N_4O_2$: 299.1430; found 299.4. Anal. Calcd for $C_{16}H_{18}N_4O_2$ (298): C, 64.41; H, 6.08; N, 18.78. Found: C, 64.53; H, 6.10; N, 18.83.

4.7.3. **2-(2-ethoxyphenyl)-5-methyl-7-propyl-imidazo[5,1-*f*][1,2,4]triazin-4(*3H*)-one 14c**. The title compound was prepared from 0.26 g (0.0008 mol) of **13c**. The final product was crystallised from ethyl acetate to afford 0.19 g (78 %) of **14c** as a light yellow solid, mp 142 -144 ºC. ^{1}H NMR ($CDCl_3$, δ ppm): 9.98 (brs, 1H, NH···O), 8.17-8.15 (dd, *J* = 1.5 and 7.8 Hz, 1H, 6-H of C_6H_4), 7.51-7.48 (t, *J* = 8.5 Hz, 1H, 4-H of C_6H_4), 7.14-7.11 (t, *J* = 7.6 Hz, 1H, 5-H of C_6H_4), 7.05(d, *J* = 8.3 Hz, 1H, 3-H of C_6H_4), 4.28-4.24 (q, *J* = 7 Hz, 2H, CH_2O), 3.02-2.99 (t, *J* = 7.6 Hz, 2H, CH_2C), 2.64 (s, 3H,CH_3C), 1.90-1.86 (m, *J* = 7.5 Hz, 2H, CH_2C**H**$_2$$CH_3$), 1.58-1.55 (t, *J* = 7.1 Hz, 3H, C**H**$_3$$CH_2O$), 1.04-1.01 (t, *J* = 7.3 Hz, 3H, C**H**$_3$$CH_2C$). ^{13}C NMR ($CDCl_3$, δ ppm): 157.2, 155.2, 146.3, 146.1, 139.9, 133.3, 130.3, 121.9, 117.8, 114.1, 113.3, 65.5, 28.2, 21.2, 14.9, 14.7, 14.2. MS-ESI (matrix CCA): *m/z* $[M+H]^+$ calcd for $C_{17}H_{20}N_4O_2$: 313.1586; found 313.3. Anal. Calcd for $C_{17}H_{20}N_4O_2$ (312): C, 65.37; H, 6.45; N, 17.94. Found: C, 65.45; H, 6.43; N, 17.88.

4.7.4. **2-(2-ethoxyphenyl)-5-methyl-7-phenyl-imidazo[5,1-*f*][1,2,4]triazin-4(*3H*)-one 14d**. The title compound was prepared from 0.37 g (0.001 mol) of **13d**. The final product was crystallised from ethyl acetate to afford 0.27 g (77 %) of **14d** as a light yellow solid, mp 181-182 ºC. ^{1}H NMR ($CDCl_3$, δ ppm): 10.18 (brs, 1H, NH···O), 8.40-8.38 (d, *J* = 7.8 Hz, 2H, C_6H_5), 8.19-8.17 (d, *J* = 8.3 Hz, 1H, 6-H of C_6H_4), 7.53-7.49 (q, *J* = 7.2 Hz, 3H, C_6H_5), 7.46-7.44 (t, *J* = 7.1 Hz, 2H, 4-H of C_6H_4), 7.15-7.12 (t, *J* = 7.6 Hz, 1H, 5-H of C_6H_4), 7.05-7.04 (d, *J* = 8.3 Hz, 1H, 3-H of C_6H_4), 4.28-4.24 (q, *J* = 6.8 Hz, 2H, CH_2O), 2.74 (s. 3H, CH_3C), 1.59-1.56 (t, *J* = 7.1 Hz, 3H, C**H**$_3$$CH_2O$). ^{13}C NMR ($CDCl_3$, δ ppm): 157.3, 155.0, 146.9, 142.4, 141.0, 133.5, 130.3, 129.7, 129.0, 128.7, 122.0, 117.4, 115.5, 113.2, 65.5, 14.9, 14.8. MS-ESI (matrix CCA): *m/z* $[M+H]^+$ calcd for $C_{17}H_{20}N_4O_2$: 346.1430; found 347.2. Anal. Calcd for $C_{20}H_{18}N_4O_2$ (346): C, 69.35; H, 5.24; N, 16.17. Found: C, 69.55; H, 5.26; N, 16.21.

4.7.5. **2-(2-ethoxyphenyl)-5-methyl-7-carboethoxy-imidazo[5,1-*f*] [1,2,4]triazin-4(*3H*)-one 14e**. The title compound was prepared from 1.0 g (0.0028 mol) of **13e**. Crystallisation from ethyl acetate afforded 0.69 g (73 %) of **14e** as a light yellow solid, mp 214-216 ^{o}C; R_f (hexane/ EtOAc, 1:1) 0.31. ^{1}H NMR ($CDCl_3$, δ ppm): 10.56 (brs, 1H, NH···O), 8.42-8.4 (d, *J* = 7.8 Hz, 1H, 6-H of C_6H_4), 7.57-7.54 (t, *J* = 7.8 Hz, 1H, 4-H of C_6H_4), 7.19-7.16 (t, *J* = 7.6 Hz, 1H, 5-H of C_6H_4), 7.08 (d, *J* = 8.8 Hz, 1H, 3-H of C_6H_4), 4.58-4.54 (q, *J* = 7 Hz, 2H, CH_2O), 4.34-4.3 (q, *J* = 7 Hz, 2H, CH_2O), 2.74 (s, 3H, CH_3C), 1.63-1.6 (t, *J* = 7 Hz, 3H, C**H$_3$**CH_2O), 1.53-1.5 (t, *J* = 7.1 Hz, 3H, C**H$_3$**CH_2O). ^{13}C NMR ($CDCl_3$, δ ppm): 157.54, 157.48, 154.4, 148.4, 141.2, 134.0, 132.8, 130.8, 122.3, 117.8, 116.7, 113.2, 65.7, 62.2, 14.9, 14.8, 14.6. MS-ESI (matrix CCA): *m/z* $[M+H]^+$ calcd for $C_{17}H_{18}N_4O_4$: 343.1328; found 343.2. Anal. Calcd for $C_{17}H_{18}N_4O_4$ (342): C, 59.64; H, 5.30; N, 16.37. Found: C, 59.65; H, 5.32; N, 16.31.

*4.8. 2-[(2-ethoxy-5(4-ethylpiperazine-1-sulphonyl)phenyl]-7-etoxycarbonyl-5-methyl-imidazo[5,1-f][1,2,4]triazin-4(3H)-one **15e***

2-(2-ethoxyphenyl)-7-ethyl-5-methyl-imidazo[5,1-*f*][1,2,4]triazin-4(*3H*)-one **14e** (200 mg, 5.84×10^{-4} mol) was slowly added to 0.5 mL (874.5 mg; 7.5×10^{-4} mol) of chlorosulphonic acid. The reaction mixture was stirred for 1.5 h at room temperature. The product was poured into ice-water (5 ml) and extracted with dichloromethane (3×15 ml). The chloroform extracts were combined, dried over anhydrous magnesium sulphate, filtered and the solvent was evaporated to give 234 mg of a benzenosulphonyl chloride white powder; yield 92 %; R_f (EtOAc/EtOH 6:1) 0.75; R_f (EtOAc) 0.7. Benzenosulphonyl chloride (234 mg, 5.32×10^{-4} mol) was dissolved in 3 ml of THF and cooled to 0 ºC. *N*-Ethylpiperazine (133.7 mg, 0.15 ml; 1.17×10^{-3} mol) was added to this solution. The reaction mixture was stirred at room temperature for 18 h. The solvent was evaporated *in vacuo* and the residue was dissolved in dichloromethane (5 mL). The organic solution was washed twice with water, dried over anhydrous magnesium sulphate, filtered and the solvent was evaporated. The residue was purified by column chromatography on silica gel (developed with hexane/EtOAc, 1:1 v/v) afforded the product as a light yellow solid which was crystallised from abs. ethanol to give 170 mg (62 %), mp 215–7 ºC; R_f (EtOAc/EtOH, 6:1) 0.23; R_f (EtOAc) 0.1. ^{1}H NMR ($CDCl_3$, δ ppm): 10.18 (bs, 1H, NH···O), 8.69 (d, *J* = 2.4 Hz, 1H, 6-H of C_6H_4), 7.93-7.91 (dd, *J* = 2.4 and 8.8 Hz, 1H, 4-H of C_6H_4), 7.21-7.19 (d, *J* = 8.8 Hz, 1H, 3-H of C_6H_4), 4.56-4.52 (q, *J* = 7.2 Hz, 2H, CH_2O), 4.42-4.38 (q, *J* = 7 Hz, 2H, CH_2O), 3.21 (bs, 4H, 2×CH_2N), 2.73 (s, 3H, CH_3C), 2.67 (bs, 4H, 2×CH_2N), 2.53 (bs, 2H, NC**H$_2$**CH_3), 1.66-1.63 (t, *J* = 6.8 Hz, 3H, CH_3), 1.53-1.5 (t, *J* = 7.1 Hz, 3H, CH_3), 1.12 (bs, 3H, NCH_2C**H$_3$**). ^{13}C NMR: ($CDCl_3$, δ ppm): 157.5, 157.5, 154.4, 148.4, 141.2, 134.0, 132.8, 130.8, 128.5, 117.8, 116.7, 113.2, 65.7, 62.2, 54.2, 48.4, 45.8, 14.9, 14.6, 13.3. MS-ESI (matrix CCA): *m/z* $[M+H]^+$ calcd for $C_{23}H_{30}N_6O_6S$: 518.20; found 519.3. Anal. Calcd for $C_{23}H_{30}N_6O_6S$ (518): C, 53.27; H, 5.83; N, 16.21; S, 6.18. Found: C, 53.45; H, 5.85; N, 16.25; S, 6.20.

*4.9. ethyl 3-bromo-2-oxo-butyrate **16***

This compound was prepared from 20 g (0.0895 mol) $CuBr_2$ and 7.15 g (0.055 mol) ethyl 2-oxobutanoate according to the method reported by Okonya [26] in 87 % yield (lit.[26] yield 80 %); ^{1}H NMR ($CDCl_3$, δ ppm): 5.20-5.16 (q, *J* = 6.8 Hz, 1H, CHBr), 4.42-4.36 (m, 2H, CH_2O), 1.82-1.81 (d, *J* = 6.8 Hz, 3H, CH_3CH), 1.42-1.39 (t, *J* = 7.1 Hz, 3H, CH_3CH_2).

Acknowledgments

The authors thank Dr. J. Chojnacki for the X-ray structure determination. The partial financial support of these studies by the pharmaceutical company Polpharma SA is gratefully acknowledged.

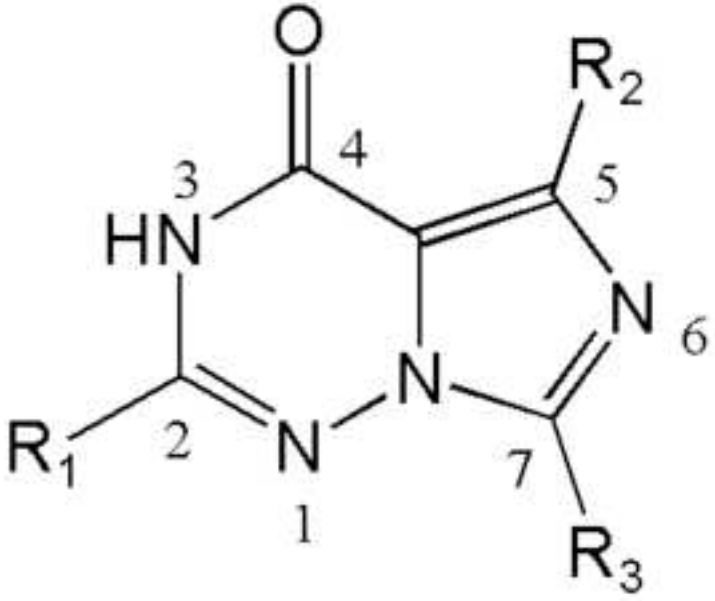
References and notes

1. Beavo, J. A. *Physiol. Rev*. **1995**, *75*, 725-748.
2. Jeon, Y. H.; Heo, Y. -S.; Kim, C. M.; Hyun, Y. -L.; Lee, T. G.; Ro, S.; Cho, J. M. *Cell. Mol. Life Sci.* **2005**, *62*, 1198-1220.
3. Zagrovic, B.; van Gunsteren, W. F. *J. Chem. Theory Comput*. **2007**, *3*, 301-311.
4. Sung, B-J.; Hwang, K. Y.; Jeon, Y. H.; Lee, J. I.; Heo, Y-S.; Kim, J. H.; Moon, J.; Yoon, J. M.; Hyun, Y-L.; Kim, E.; Eum, S. J.; Park, S-Y.; Lee, J-O.; Lee, T. G.; Ro, S.; Cho, J. M. *Nature* **2003**, *425*, 98-102.
5. Corona, G.; Mondaini, N.; Ungar, A.; Rozzoli, E.; Rossi, A.; Fusco, F. *J. Sexual Medicine* **2011**, *8*, 3418-3432.
6. Kim, D-K.; Lee, N.; Lee, J. Y.; Ryu, D. H.; Kim, J-S.; Lee, S-H.; Choi, J-Y.; Chang, K.; Kim, Y-W.; Im, G-J.; Choi, W-S.; Kim, T-K.; Ryu, J-H.; Kim, N-H.; Lee, K. *Bioorg. Med. Chem.* **2001**, *9*, 1609-1616.
7. Yu, G.; Mason, H.; Wu, X.; Wang, J.; Chong, S.; Beyer, B.; Henwood, A.; Pongrac, R.; Seliger, L.; He, B.; Normandin, D.; Ferrer, P.; Zhang, R.; Adam, L.; Humphrey, W. G.; Krupinski, J.; Macor, J. E. *J. Med. Chem*. **2003**, *46*, 457-460.
8. Dell'Agli, M.; Galli, G. V.; Cero, E. D.; Belluti, F.; Matera, R.; Zironi, E.; Pagliuca, G.; Bosisio, E. *J. Nat. Prod*. **2008**, *71*, 1513-1517.
9. Eardley, I. *Exp. Opin. Invest. Drugs* **1997**, *6*, 1803-1810.
10. Ishibashi, Y.; Matsui, T.; Takeuchi, M.; Yamagishi, S. *Clin. Exp. Med*. **2011**, *11*, 131-135.
11. Jing, Z. C.; Yu, Z. X.; Shen, J. Y.; Wu, B. X.; Xu, K. F.; Zhu, X. Y.; Pan, L.; Zhang, Z. L.; Liu, X. Q.; Zhang, Y. S.; Jiang, X.; Galie. N. *Am. J. Respir. Crit. Care Med.* **2011**, *183*, 1723-1729.
12. Dunn, P. J. *Org. Proc. Res. Develop.***2005**, *9*, 88-97.
13. Haning, H.; Niewöhner, U.; Schenke, T.; Es-Sayed, M.; Schmidt, G.; Lampe, T.; Bischoff, E. *Bioorg. Med. Chem. Lett*. **2002**, *12*, 865-868.
14. Seidel, D. *J. Labelled Compd. Radiopharm.* **2001**, *44*, 961-962.
15. Seidel, D.; Brehmer, P.; Schoof, Y.; Weinberg, U.; Niewöhner, U.; Nowakowski, M. *J. Labelled Compd. Radiopharm.* **2003**, *46*, 1019-1032.
16. Heim-Riether, A.; Healy, J. *J. Org. Chem*. **2005**, *70*, 7331-7337.
17. Charles, I.; Latham, D. W. S.; Hartley, D.; Oxford, A. W.; Scopes, D. I. C. *J. Chem. Soc., Perkin Trans. 1* **1980**, 1139-1146.
18. Dakin, H. D.; West, R. *J. Biol. Chem*., **1928**, *78*, 91.
19. Tran, K-V.; Bickar, D. *J. Org. Chem*. **2006**, *71*, 6640-6643.
20. Garigipati, R. S. *Tetrahedron Lett*. **1990**, *31*, 1969-1972.
21. Mao, Y.; Tian, G.; Liu, Z.; Shen, J.; Shen, J. *Org. Proc. Res. Develop.* **2009**, *13*, 1206-1208.
22. Niewöhner, U.; Es-Sayed, M.; Haning, H.; Schenke, T.; Schelmmer, K-H.; Keldenich, J.; Bischoff, E.; Perzborn, E.; Dembowsky, K; Serno, P.; Nowakowski, M.PTC Int. Appl. WO 1999 24433, 1999; *Chem. Abst*. **1999**, *130*, 352283.
23. Nowakowski, M.; Gehring, R.; Heilmann, W.; Wahl, K-H. PTC Int. Appl. WO 2002 50076, 2002; *Chem. Abst.* **2002**, *137*, 47233.
24. Weintraub, L.; Oles, S. R.; Kalish, N. *J. Org. Chem*. **1968**, *33*, 1679-1681.
25. Nakamura, K.; Inoue, K.; Ushio, K.; Oka, S.; Ohno, A. *J. Org. Chem*. **1988**, *53*, 2589-2593.
26. Okonya, J. F.; Hoffman, R. V.; Johnson, M. C. *J. Org. Chem.* **2002**, *67*, 1102-1108.

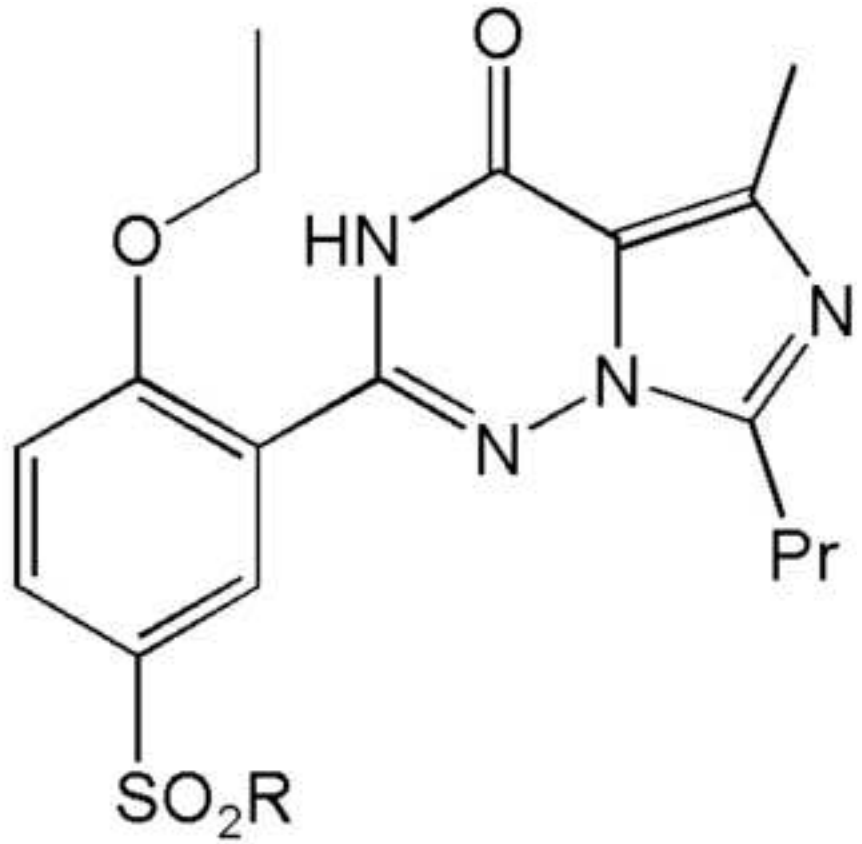
Supplementary Material

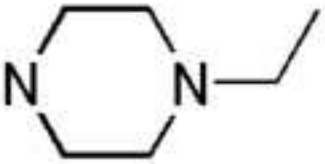
NMR spectra (1H and 13C) for all products. This material is available free of charge.

Figure(s) (use if uploading high quality figure files)
Click here to download high resolution image



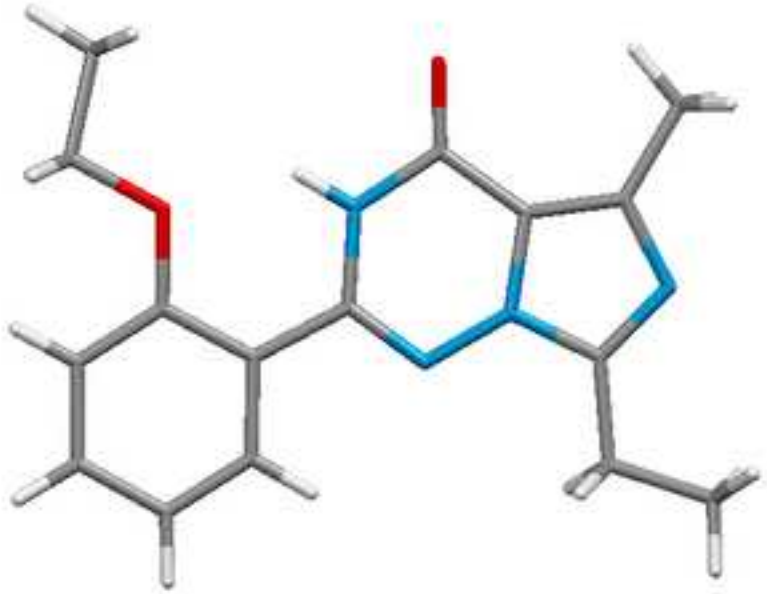
1



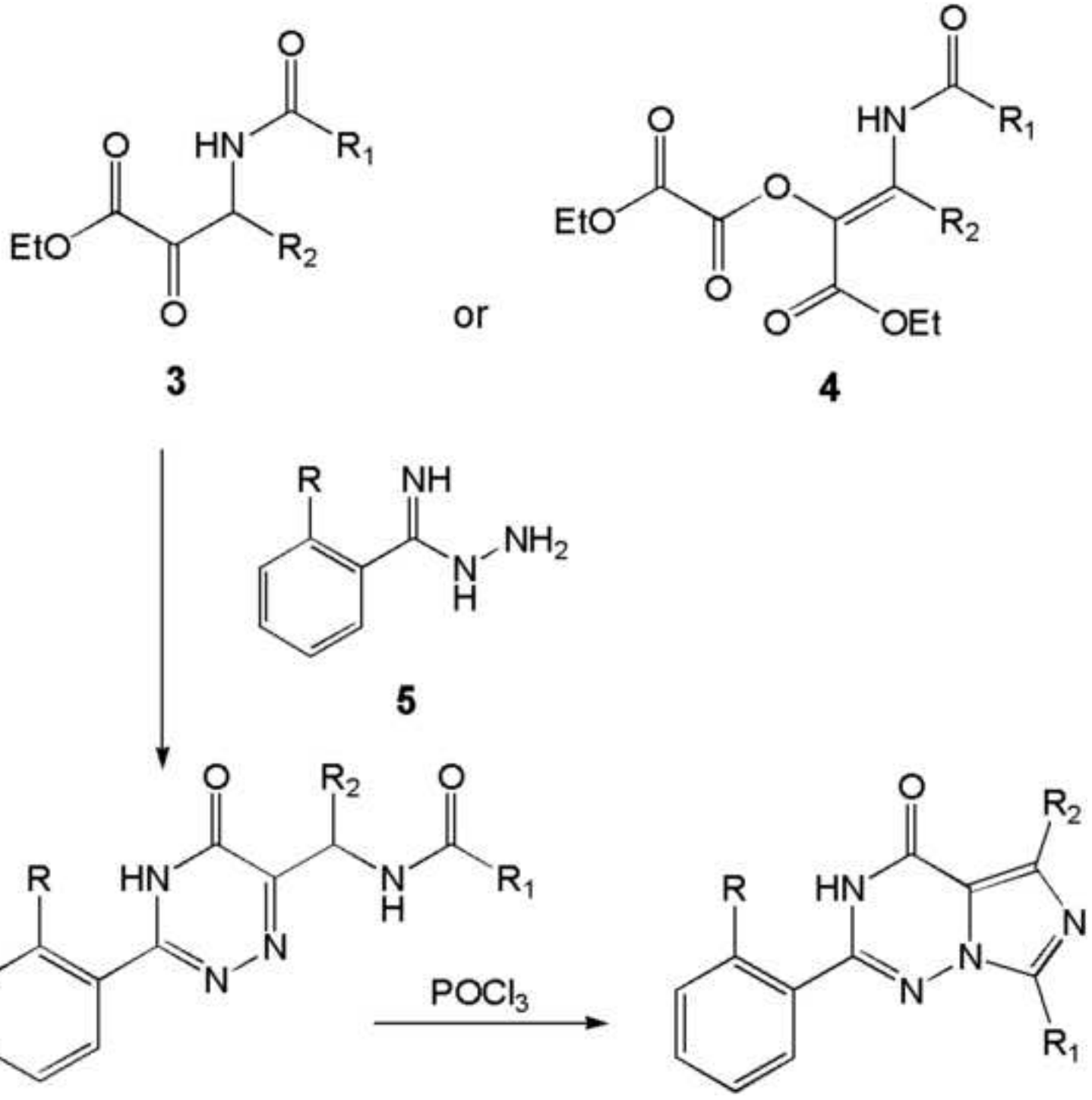
2 R = 

Figure(s) (use if uploading high quality figure files)

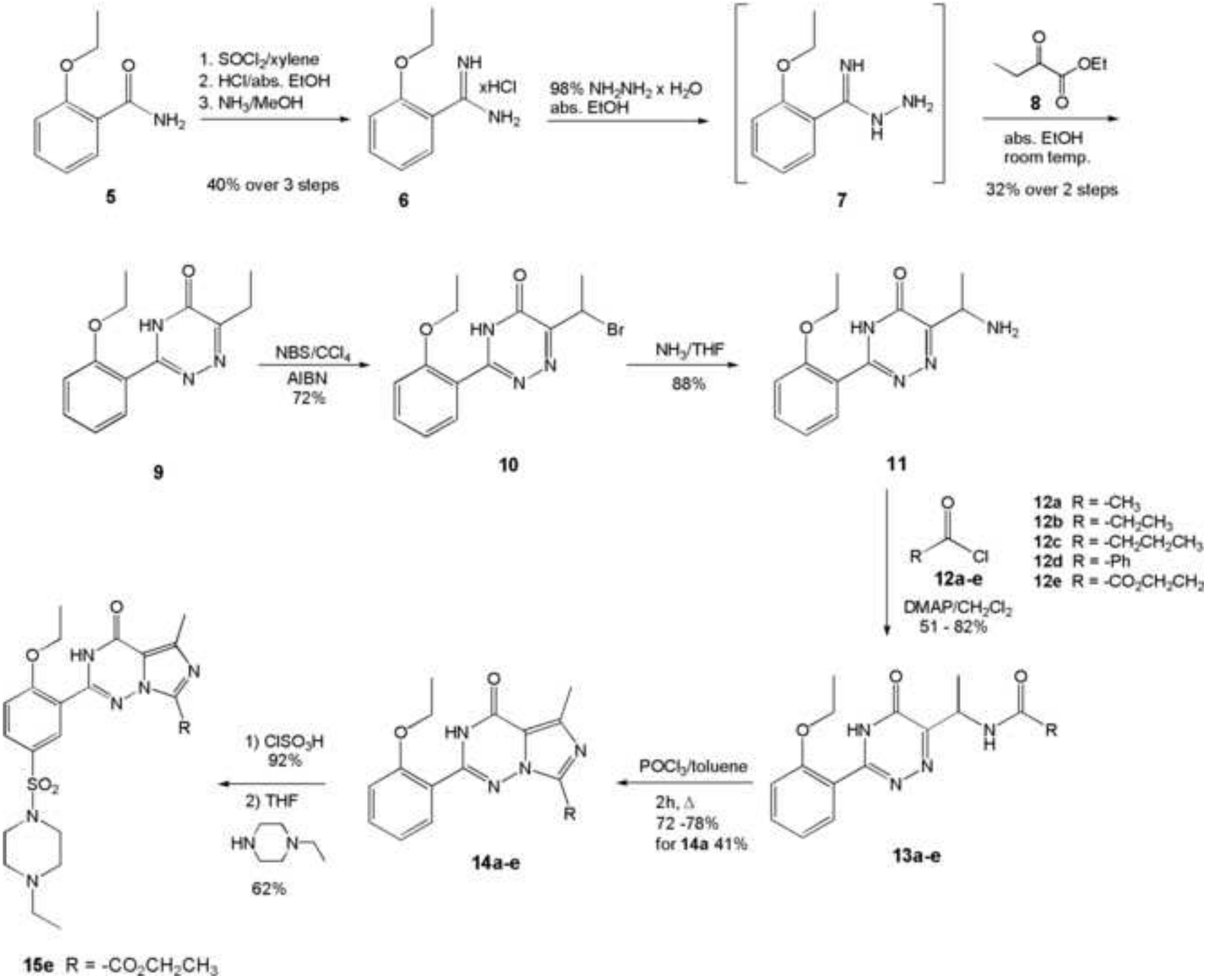
Click here to download high resolution image



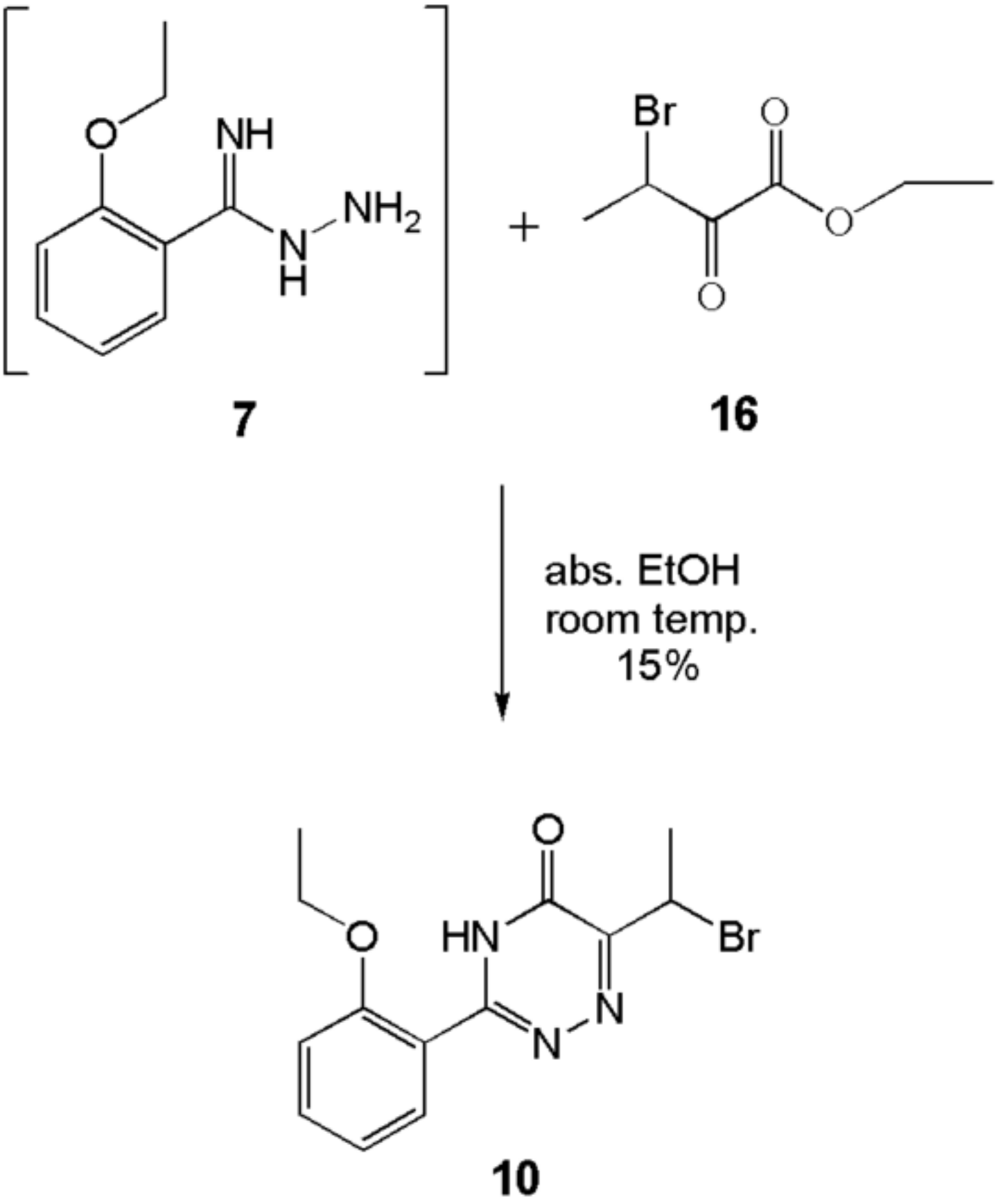
Figure(s) (use if uploading high quality figure files)
Click here to download high resolution image



Figure(s) (use if uploading high quality figure files)
Click here to download high resolution image



Figure(s) (use if uploading high quality figure files)
Click here to download high resolution image



7 **16**

10