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Annelated pyrrolo-pyrimidines from amino-cyanopyrroles and BMMAs as leads for new DNA-interactive ring systems

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Abstract—The efficient one-pot synthesis of several new tricyclic systems of type **1** and **2**, obtained from the reaction of substituted 2-amino-3-cyanopyrroles and 3-amino-4-cyanopyrroles with BMMAs, is reported. The duration and yields of the reaction strongly depend on the reactivity of the starting pyrrole and on the size of the ring to be formed. Mechanist features of the reaction were investigated and proposed by studying also the reactivity of a 3-aminopyrrole-2,4-dicyano substituted. The method reported represents the first example of the use of BMMA reagents in combination with pyrrole derivatives and allows an easy and versatile entry to a large number of hitherto unknown pyrrolo-pyrimidines further annelated with nitrogen heterocycles of different sizes. These new polycondensed heterocycles possess the requisite to interact with DNA.

1. Introduction

In the search for new anticancer drugs different approaches are currently undertaken. Several efforts are directed towards the discovery of molecules targeting angiogenesis, cell-cycle pathways, and cell differentiation. However classical approaches involving the discovery of cytotoxic agents interfering with DNA, either directly or inhibiting DNA-binding enzymes, have led to the identification of new promising anticancer agents.[1]

As our aim is continuously devoted to the study of new flat polycyclic heteroaromatic systems that can behave as DNA-interactive drugs, we have successfully prepared and reported the antiproliferative activity of several tricyclic and tetracyclic heterocycles that incorporate the pyrrole or indole moieties. Among them indolo[3,2-*c*]cinnolines,[2] indolo[1,2-*c*]benzo[1,2,3]triazines,[3] pyrrolo[3,4-*e*][1,2,3]triazolo[1,5-*a*]pyrimidines,[4] pyrrolo[2,3-*e*][1,2,3]triazolo[1,5-*a*]pyrimidines,[5] and indolo[3,2-*e*][1,2,3]triazolo[1,5-*a*]pyrimidine[6] can be related to the well known classes of intercalators with linear or angular structure such as acridines, anthracyclines, and phenanthridines. For these compounds the principal driving forces are stacking and charge-transfer interactions as well as hydrogen bonding and electrostatic forces.[7] However in some cases intercalation into DNA is only the first step in the events that eventually lead to DNA damage by other mechanisms. Moreover if the drug candidate bears suitable substituents, such as a thiomethyl group, interference with cellular detoxification pathways can be envisaged.

Therefore in search of suitable lead compounds for the development of new anticancer drugs, we decided to explore series of new tricyclic heterocycles as DNA-interactive agents and to investigate synthetic methods leading to annelated pyrrolo-pyrimidines.

Few years ago the use of new reagents for heterocyclic annelation containing an *N*-[bis(methylthio)methylene]amino moiety (= BMMA) was reported. The method allowed the preparation of condensed pyrimidines in a one-pot reaction from (hetero)aromatic *ortho*-aminocarbonyl-type compounds ('carbonyl' = COOEt, COMe, and CN).[8] Some authors showed the use of BMMAs in combination with electron-rich pentatomic heterocycles (furan[9] and thiophene[10]) and their condensed homologues, but nothing has been reported so far on their employment in reactions with pyrroles

Keywords: Annelated pyrrolo-pyrimidines; Amino-cyanopyrroles; BMMA reagent; DNA-interactive polycycles.
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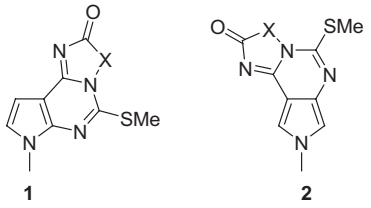
or indoles. However our experience on the behavior of aminopyrroles and aminoindoles[11] suggested that the electron-rich pyrrole and indole should be able to react with this kind of reagents as well and prompted us to investigate their use in combination with BMMAs as an access to hitherto unknown annelated pyrrolo-pyrimidines.

Therefore, in this paper we report the efficient one-pot synthesis of several new tricyclic systems of type **1** and **2**, obtained from the reaction of substituted 2-amino-3-cyanopyrroles and 3-amino-4-cyanopyrroles with several BMMA reagents.



2. Results and discussion

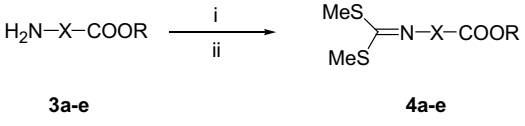
The starting BMMA reagents **4a**–**e** were prepared in good yields by reacting adequate amino educts **3** with CS_2/MeI/NEt_3 and subsequently accomplishing the alkylation of the intermediates thus obtained with MeI/K_2CO_3 (Scheme 1).

The feasibility of the reaction with BMMAs was first investigated by using 2-amino-3-cyanopyrroles as substrates. For this purpose the pyrrole derivatives **5a**–**c** were prepared by standard procedures[12] and were allowed to react with *N*-[bis(methylthio)methylene]glycine ethyl ester (**4a**) in acetic acid under reflux (Scheme 2). The starting materials were converted into the final products in 30 min. The imidazo[1,2-*c*]pyrrolo[3,2-*e*]-pyrimidinones **6a**,**b** were isolated in very high yields (85–90%), only in the case of the aminopyrrole **5c**, the tricyclic compound **6c** was obtained in 40% yield because the competing alkylation of the unsubstituted α-position of the pyrrole ring gave rise to the formation of a large amount of derivative **7** (35%).

This first approach showed that the BMMAs can be used to annelate the pyrrole nucleus and that the reaction spontaneously proceeded to the final products through a sequence that can be envisaged as a domino reaction in which the primary cyclized bicycle (the pyrrolo-pyrimidine **8**), because of the presence of a reactive imine, further cyclizes originating the third ring (Scheme 3). Moreover the 2-amino-3-cyanopyrrole resulted much more reactive toward BMMAs than the aryl or thiophene substrate already studied in literature (shorter reaction times and higher yields).[8] However the method was not suitable for usage with the pyrrole unsubstituted in the α-position and therefore the reactivity of derivative **5c** was not investigated any more.

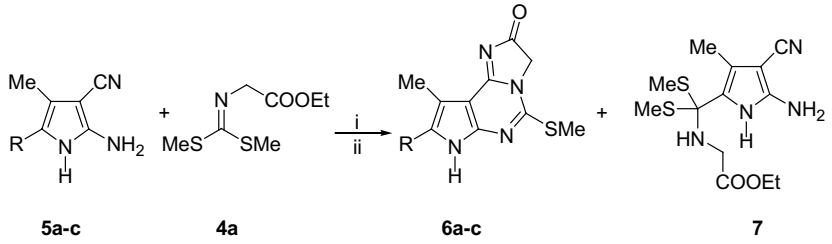
2-Amino-3-cyanopyrroles **5a**,**b** were allowed to react with all the other BMMAs **4b**–**e** in acetic acid under reflux for an appropriate period of time (0.5–1 h). In all the cases the annelated pyrrolo-pyrimidines **9**–**12** could be easily isolated generally in yields from good to high (Scheme 4).

Only in the case of the reaction with **4d** the yields resulted lower probably because of steric hindrance in the intermediate **8** (X = CHMe) which can not properly assume a conformation suitable for the ring closure (compare **6** vs **11**, yields 90–85% vs 60–43%). On the

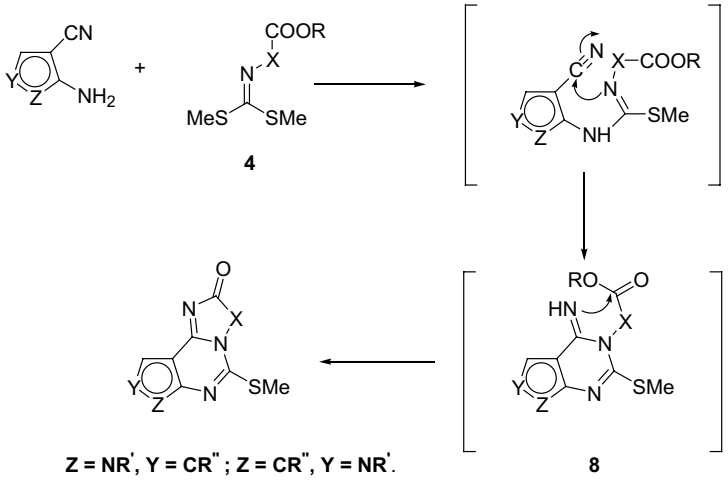


	X	R
a	CH_2	Et
b	CH_2CH_2	Et
c	NMe	Et
d	CHMe	Me
e	$CHMeCH_2$	Et

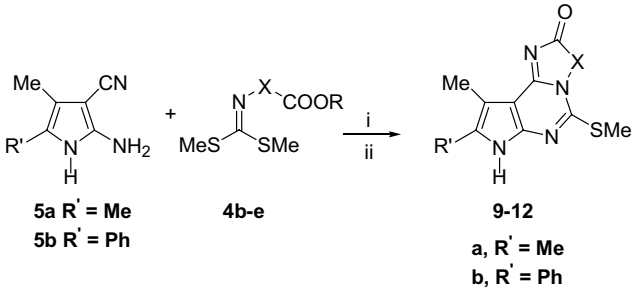
Scheme 1. Reagents: (i) CS_2, MeI, NEt_3, $CHCl_3$; (ii) MeI, K_2CO_3, Me_2CO.



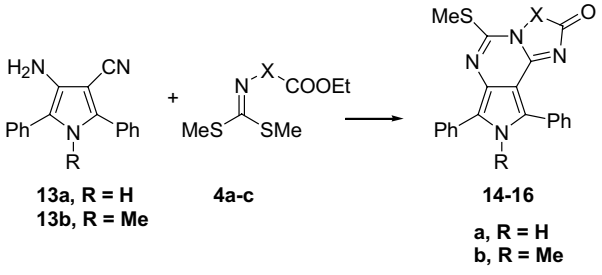
Scheme 2. (a) R = Me; (b) R = Ph; (c) R = H. Reagents: (i) AcOH, Δ; (ii) $NaHCO_3$ or Na_2CO_3.



Scheme 3. Mechanism of formation of annelated pyrrolo-pyrimidines.



Scheme 4. Reagents: (i) AcOH, Δ; (ii) $NaHCO_3$ or Na_2CO_3.



Scheme 5. Reagents: (i) AcOH, Δ; (ii) $NaHCO_3$ or Na_2CO_3.

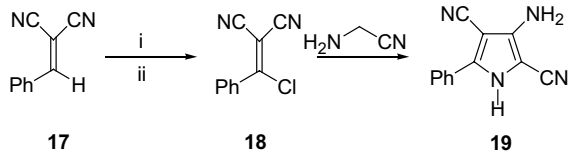
other hand in the homologous intermediate (X = $CHMeCH_2$) the formation of the six-membered ring is easier, as testified by the higher yields, and a shorter reaction time is required (compare **11a** vs **12a**, **11b** vs **12b**).

Thus the reaction of 2-amino-3-cyanopyrroles with BMMAs seemed to be of general application. Therefore we decided to investigate the behavior of 3-amino-4-cyanopyrroles. For this purpose the 3-amino derivatives **13a**,**b** were prepared by known procedure[13] and were allowed to react with the BMMAs **4a**–**c** (Scheme 5).

Once again the tricyclic derivatives **14**–**16** were isolated after heating in acetic acid under reflux for the appropriate reaction time (4–8 h). It is worthy to note that in this case the time required to drive the reaction to completion is longer and the yields are generally lower if compared to those obtained in the case of the reaction with 2,3-disubstituted pyrroles. Therefore it seems that the annelation on the position 2 and 3 of the pyrroles is favored with respect to the ring closure between positions 3 and 4 and that the rate limiting step of the domino reaction is the formation of the pyrimidine leading to the bicyclic intermediates of type **8** (Scheme 3). This is not surprising since it is well known that in five-membered electron-rich heterocycles there is a larger transmission of electronic effects between the 2- and 3-positions (hyper-*ortho*) than between the 3- and 4-positions (hypo-*ortho*) due to the high 'bond fixation', which gives rise to C-2–C-3 bond shorter and with a high π bond order.[11b,14] To verify how this effect could influence the course of the reaction we decided to investigate the two possible ring closures in competition by using as starting material the 3-amino-2,4-dicyanopyrrole **19**. This last was suitably prepared by a modification of the method reported in literature[15] (Scheme 6), from benzylidenemalonitrile **17**.

The reaction of the pyrrole **19** with the BMMA **4a** led to a mixture of the annelated pyrrolo-pyrimidines **21** and **23**, as expected (Scheme 7). The main product of the reaction was the imidazo-pyrrolo-pyrimidine **23** (yield 40%), derived from ring closure on the 2- and 3-positions, confirming thus that the key step of the reaction sequence is the formation of the intermediate bicycle and that **22** is formed more efficiently than **20**, since it is reasonable to suppose that there is no significant difference in the ring closure originating the third ring.

Of course the structure of derivatives **21** and **23** was assigned especially on the basis of NMR spectra and in comparison with the data obtained for the other isomeric compounds synthesized herein (**6a**–**c**, **14a**,**b**). In particular very useful resulted the analysis of the ^{13}C chemical shifts, which were also correlated with theoretically estimated values.[16] In fact, taking into account the effect of the substituents, the chemical shifts of the carbon atoms present analogous values in the two series in which the pyrrole nucleus is annelated in the same



Scheme 6. Reagents: (i) Cl_2, PyHCl; (ii) NEt_3.

positions (compare **6b** and **23**, **14a** and **21**) and a good correlation was found between calculated and found values in the case of derivatives **21** and **23** ($r^2 = 0.749$ and 0.926, respectively).

Representative compounds of each series were selected for screening tests by the NCI (Bethesda) in the Developmental Therapeutics Program. In the primary anticancer screening assay,[17] tested against a 3-cell line panel consisting of the MCF7 (Breast), NCI-H460 (Lung), and SF-268 (CNS) the annelated pyrrolo-pyrimidine resulted generally inactive up to 10^{-4} M concentrations. However tested against the human intestinal adenocarcinoma (LoVo),[18] although being less active than the reference drug [doxorubicin (DOXO)], few compounds exhibited a moderate antiproliferative activity with IC_{50} = 10.5–41.2 μM (Fig. 1).

From the data shown in Figure 1 it is worthy to note that generally the triazolo-pyrimidine derivatives resulted inactive or low active regardless to the condensation position (compare **10b** and **16a**), whereas the presence of the imidazole ring enhances the antiproliferative activity, especially in the case of pyrimidine moiety condensed on the positions 3 and 4 of the pyrrole nucleus (compare derivatives **6** and **14**). Ring enlargement to give the pyrimido-pyrimidine originates the most active compound of these series, derivative **15a**. In order to evaluate the potential ability of the new annelated pyrrolo-pyrimidine ring systems to interact with DNA, we calculated[19] the LUMO and HOMO energies, considering that these variables are of importance when two molecules with π electron systems form charge-transfer complexes, and also the values of some molecular descriptors [molecular surface area, ASA (accessible surface area), log *P*, molar refractivity].

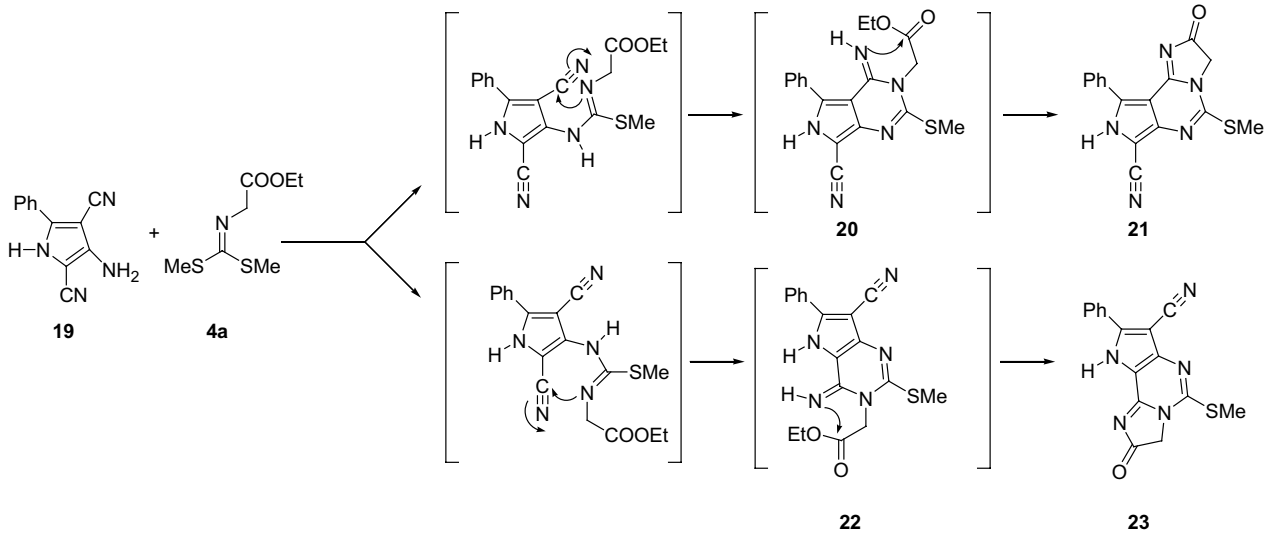
An analysis of the data reported in Table 1 shows that these values are comparable with those of well known DNA-intercalators of the acridine or anthracycline classes such as amsacrine (AMSA) and doxorubicin (DOXO). Moreover they share the same features of other tetracyclic systems, which already demonstrated to be active against a wide range of tumor cell lines, the indolo[1,2-*c*]benzo[1,2,3]triazine[3] (TRIAZINE) and the indolo[3,2-*c*]cinnoline[2] (CINNOLINE). Therefore these pyrrolo-pyrimidine derivatives appear to be good candidate as DNA-interactive drugs.

3. Conclusions

This method represents the first example of the use of BMMA reagents in combination with pyrrole derivatives and allow an easy and versatile entry to a large number of hitherto unknown pyrrolo-pyrimidines further annelated with nitrogen heterocycles of different sizes. Preliminary structure activity analysis on these new classes of tricyclic heterocycles demonstrated that although not being very active, derivatives of these systems possess the electronic and steric requisite to interact with DNA and could constitute suitable lead compounds for new anticancer agents.

4. Experimental

All melting points were taken on a Buchi–Tottoli capillary apparatus and are uncorrected; IR spectra were determined in bromoform with a Jasco FT/IR 5300 spectrophotometer; ^1H and ^{13}C NMR spectra were measured in DMSO-d_6 solution, unless otherwise specified, (TMS as internal reference) at 200 and 50.3 MHz,



Scheme 7. Reaction conditions: AcOH, Δ.

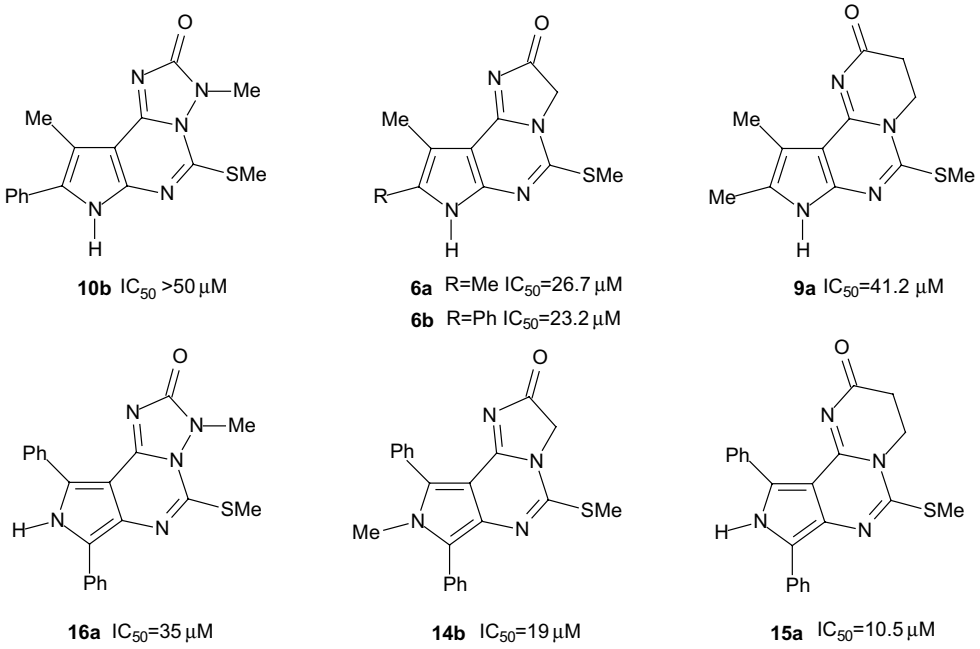


Figure 1. Antiproliferative activity against LoVo cell line[18] of annelated pyrrolo-pyrimidines.

Table 1. Molecular descriptors for annelated pyrrolo-pyrimidines

Compound	Molecular surface area[a]	ASA[a]	Log P[b]	Molar refractivity[c]	E_{LUMO}[d]	E_{HOMO}[d]
6a	234.32	258.18	1.9052	67.506	−0.8254	−8.4371
6b	283.78	318.76	3.3393	87.370	−0.8615	−8.4130
9a	248.57	278.48	2.0865	72.205	−0.7523	−8.3312
10b	297.18	332.65	NC	NC	−1.0205	−8.5312
14b	342.48	404.43	4.6245	112.99	−0.6532	−8.4339
15a	335.78	395.29	4.5593	112.79	−0.7787	−8.2797
16a	341.85	385.46	NC	NC	−1.1165	−8.3655
TRIAZINE	232.31	262.23	4.5885	80.581	−1.5652	−8.9688
CINNOLINE	235.63	258.17	2.5766	74.209	−1.4278	−9.2250
DOXO	458.18	524.23	0.1718	134.02	−1.4032	−8.9670
AMSA	344.32	398.26	3.1631	107.77	−1.2618	−8.1802

[a] (Å^2).
[b] Predicted values were calculated as a sum of the atomic values determined (see Ref. 20).
[c] (Å^3).
[d] (eV); NC: not calculated.

respectively, using a Bruker AC-E series 200 MHz spectrometer. Column chromatography was performed with Merck silica gel 230–400 mesh ASTM or with a Biotage FLASH40i chromatography module (prepacked cartridge system).

Glycine ethyl ester hydrochloride (**3a**), β-alanine ethyl ester hydrochloride (**3b**), ethyl carbazate (**3c**), L-alanine methyl ester hydrochloride (**3d**), and ethyl 3-aminobutyrate (**3e**) were purchased from Aldrich and used without further purification.

4.1. General procedure for the synthesis of BMMA reagents 4a–e

The amino ester **3a**–**e** (0.5 mol), CS_2 (40.0 g, 0.5 mol), and triethylamine (106.0 g, 1.050 mol) were reacted at 40 °C in chloroform (500 mL) for 1 h. MeI (177.2 g, 1.250 mol) was added and the mixture was heated under reflux for 1 h. The solution was cooled, washed with water (100 mL), and the organic layer was evaporated. The residue was dissolved in water, extracted with ether (3×100 mL) dried (Na_2SO_4), and evaporated. The crude oil was dissolved in acetone (300 mL), K_2CO_3 (100 g, 0.724 mol), and MeI (85.2 g, 0.6 mol) were added and the mixture was heated for 3 h under reflux, followed by stirring at room temperature overnight. The precipitated salt was filtered off, the acetone was removed in vacuo, water was added, and the product was extracted with ether, and dried (Na_2SO_4). Evaporation of the solvent and distillation afforded **4a**–**e** as colorless oils.

4.2. Ethyl *N*-[bis(methylsulfanyl)methylene]glycinate 4a

IR identical to an authentic sample.[9] The ^{13}C NMR not reported in literature showed: δ 14.0 (q, CH_3), 14.3 (q, CH_3), 14.0 (q, CH_3), 53.7 (t, CH_2), 60.3 (t, CH_2), 162.1 (s, C=N), 169.1 (s, CO).

4.3. Ethyl *N*-[bis(methylsulfanyl)methylene]-β-alaninate 4b

Yield 90%, IR: 1731 (CO) cm^{-1}; ^1H NMR: δ 1.18 (t, 3H, $J = 7.4$ Hz, CH_3), 2.09 (s, 6H, $2 \times CH_3$), 2.40 (t, 2H, $J = 5.9$ Hz, CH_2), 3.52 (t, 2H, $J = 5.9$ Hz, CH_2), 4.06 (q, 2H, $J = 7.4$ Hz, CH_2); ^{13}C NMR: δ 13.9 (q, $2 \times CH_3$), 14.2 (q, CH_3), 31.3 (t, CH_2), 51.2 (t, CH_2), 59.6 (t, CH_2), 157.3 (s, C=N), 172.7 (s, CO). Anal. Calcd for $C_8H_{15}NO_2S_2$: C, 43.41; H, 6.83; N, 6.33; S, 28.97. Found: C, 43.19; H, 6.98; N, 6.51; S, 29.31.

4.4. Ethyl 2-[bis(methylsulfanyl)methylene]-1-methylhydrazinecarboxylate 4c

Yield 90%, IR: 1698 (CO) cm^{-1}; ^1H NMR: δ 1.16 (t, 3H, $J = 7.1$ Hz, CH_3), 2.47 (s, 6H, $2 \times CH_3$), 2.96 (s, 3H, CH_3), 4.04 (q, 2H, $J = 7.1$ Hz, CH_2); ^{13}C NMR: δ 13.3 (q, CH_3), 14.5 (q, CH_3), 14.8 (q, CH_3), 36.0 (t, CH_2), 61.1 (q, CH_3), 154.1 (s, C=N), 174.2 (s, CO). Anal. Calcd for $C_7H_{14}N_2O_2S_2$: C, 37.82; H, 6.35; N, 12.60; S, 28.84. Found: C, 38.19; H, 6.50; N, 12.51; S, 29.11.

4.5. Methyl *N*-[bis(methylsulfanyl)methylene]alaninate 4d

Yield 80%, IR: 1739 (CO) cm^{-1}; ^1H NMR ($CDCl_3$): δ 1.43 (d, 3H, $J = 6.9$ Hz, CH_3), 2.42 (s, 3H, CH_3), 2.57 (s, 3H, CH_3), 3.72 (s, 3H, CH_3), 4.51 (q, 1H, $J = 6.9$ Hz, CH); ^{13}C NMR ($CDCl_3$,): δ 14.7 (q, CH_3), 14.8 (q, CH_3), 18.4 (q, CH_3), 51.9 (q, CH_3), 59.9 (d, CH), 161.4 (s, C=N), 172.9 (s, CO). Anal. Calcd for $C_7H_{13}NO_2S_2$: C, 40.56; H, 6.32; N, 6.76; S, 30.93. Found: C, 41.19; H, 6.40; N, 6.51; S, 31.05.

4.6. Ethyl 3-{[bis(methylsulfanyl)methylene]amino}butanoate 4e

Yield 85%, IR: 1727 (CO) cm^{-1}; ^1H NMR ($CDCl_3$): δ 1.16 (d, 3H, $J = 6.0$ Hz, CH_3), 1.23 (t, 3H, $J = 7.0$ Hz, CH_3), 2.31 (s, 3H, CH_3), 2.50 (d, 2H, $J = 6.0$ Hz, CH_2), 2.54 (s, 3H, CH_3), 4.10 (q, 2H, $J = 7.0$ Hz, CH_2), 4.17–4.28 (m, 1H, CH); ^{13}C NMR ($CDCl_3$): δ 13.9 (q, CH_3), 14.3 (q, CH_3), 14.4 (q, CH_3), 20.3 (q, CH_3), 42.8 (t, CH_2), 54.3 (d, CH), 59.8 (t, CH_2), 156.8 (s, C=N), 171.4 (s, CO). Anal. Calcd for $C_9H_{17}NO_2S_2$: C, 45.93; H, 7.28; N, 5.95; S, 27.24. Found: C, 46.19; H, 7.50; N, 6.03; S, 27.51.

4.7. General procedure for the reaction of amino-cyanopyrroles and BMMAs

The mixture of amino-cyanopyrrole **5** or **13** (3 mmol) and BMMA **4** (3 mmol) in AcOH (10 mL) was heated under reflux for the appropriate time, with stirring. After cooling, the reaction mixture was added to ice/water and neutralized with solid $NaHCO_3$ or Na_2CO_3. The solid precipitate was filtered off and crystallized or purified by chromatography.

4.8. 8,9-Dimethyl-5-(methylsulfanyl)-3*H*-imidazo[1,2-*c*]pyrrolo[3,2-*e*]pyrimidin-2(7*H*)-one 6a

From **5a** and **4a**: (reaction time 0.5 h), yields 90%; mp >300 °C (from methanol); IR: 3429 (NH), 1702 (CO) cm^{-1}; ^1H NMR: δ 2.21 (s, 6H, $2 \times CH_3$), 2.63 (s, 3H, CH_3), 4.25 (s, 2H, H-3), 11.93 (s, 1H, NH); ^{13}C NMR: δ 9.2 (q, CH_3), 10.5 (q, CH_3), 13.5 (q, CH_3), 49.6 (t, C-3), 100.6 (s, C-9a), 107.8 (s, C-9), 128.8 (s, C-8), 146.6 (s, C-6a), 150.9 (s, C-9b), 164.7 (s, C-5), 183.5 (s, C-2). Anal. Calcd for $C_{11}H_{12}N_4OS$: C, 53.21; H, 4.87; N, 22.56; S, 12.91. Found: C, 53.39; H, 4.90; N, 22.71; S, 13.04.

4.9. 9-Methyl-5-(methylsulfanyl)-8-phenyl-3*H*-imidazo[1,2-*c*]pyrrolo[3,2-*e*]pyrimidin-2(7*H*)-one 6b

From **5b** and **4a**: (reaction time 0.5 h), yields 85%; mp >300 °C (from methanol); IR: 3360 (NH), 1704 (CO) cm^{-1}; ^1H NMR: δ 2.50 (s, 3H, CH_3), 2.68 (s, 3H, CH_3), 4.31 (s, 2H, H-3), 7.36 (t, 1H, $J = 7.4$ Hz, H-4′), 7.49 (t, 2H, $J = 7.4$ Hz, H-3′ and H-5′), 7.65 (d, 2H, $J = 7.4$ Hz, H-2′ and H-6′), 12.45 (s, 1H, NH); ^{13}C NMR (pyridine-d_5): δ 11.4 (q, CH_3), 14.0 (q, CH_3), 50.0 (t, C-3), 103.0 (s, C-9a), 111.2 (s, C-9), 127.8 (d, C-4′), 127.9 (d, C-2′ and C-6′), 129.3 (d, C-3′ and C-5′), 132.2 (s, C-8), 132.8 (s, C-1′), 148.4 (s, C-6a), 152.4 (s, C-9b), 166.5 (s, C-5), 184.2 (s, C-2). Anal. Calcd for $C_{16}H_{14}N_4OS$: C, 61.92; H, 4.55; N, 18.05 S, 10.33. Found: C, 62.00; H, 4.57; N, 18.01; S, 10.51.

4.10. 9-Methyl-5-(methylsulfanyl)-3H-imidazo[1,2-*c*]-pyrrolo[3,2-*e*]pyrimidin-2(8*H*)-one 9a

From **5c** and **4a**: (reaction time 1 h); the precipitate was identified as *ethyl N-{(5-amino-4-cyano-3-methyl-1H-pyrrol-1-yl)[bis(methylsulfanyl)]}methyleneglycinate* (**7**), yields 35%; mp 160 °C (from methanol), IR: 3476–3356 (NH_2), 2925 (NH), 2206 (CN), 1741 (CO) cm^{-1}; ^1H NMR: δ 1.23 (t, 3H, $J = 7.4$ Hz, CH_3), 2.36–2.54 (m, 10H, $3 \times CH_3$ and NH), 4.18 (q, 2H, $J = 7.4$ Hz, CH_2), 4.49 (s, 2H, CH_2), 7.49 (s, 2H, NH_2), 10.35 (s, 1H, NH); ^{13}C NMR: δ 11.6 (q, CH_3), 11.7 (q, CH_3), 14.1 (q, CH_3), 18.0 (q, CH_3), 47.2 (t, CH_2), 61.1 (t, CH_2), 77.1 (s, $C(SCH_3)_2$), 112.6 (s, CN), 115.4 (s, C-3), 117.2 (s, C-4), 140.0 (s, C-2), 147.4 (s, C-5), 169.2 (s, CO). Anal. Calcd for $C_{13}H_{20}N_4O_2S_2$: C, 47.54; H, 6.14; N, 17.06; S, 19.52. Found: C, 47.49; H, 6.20; N, 17.16; S, 19.70.

The aqueous layer was extracted with EtOAc. The combined extracts were dried with anhydrous Na_2SO_4 and the solvent was removed under vacuum to give *9-methyl-5-(methylsulfanyl)-3H-imidazo[1,2-c]pyrrolo[3,2-e]-pyrimidin-2(7H)-one* (**6c**): yields 40%; mp >300 °C; IR: 3370 (NH), 1705 (CO) cm^{-1}; ^1H NMR: δ 2.28 (s, 3H, CH_3), 2.64 (s, 3H, CH_3), 4.26 (s, 2H, H-3), 6.96 (s, 1H, H-8), 11.97 (s, 1H, NH); ^{13}C NMR: δ 10.8 (q, CH_3), 13.5 (q, CH_3), 49.6 (t, C-3), 100.1 (s, C-9a), 110.2 (s, C-9), 112.8 (d, C-8), 120.0 (s, C-6a), 147.6 (s, C-9b), 151.9 (s, C-5), 183.7 (s, C-2). Anal. Calcd for $C_{10}H_{10}N_4OS$: C, 51.27; H, 4.30; N, 23.91, S, 13.69. Found: C, 51.25; H, 4.34; N, 23.99, S, 13.75.

4.11. 9,10-Dimethyl-6-(methylsulfanyl)-3,4-dihydropyrimido[1,2-*c*]pyrrolo[3,2-*e*]pyrimidin-2(8*H*)-one 9a

From **5a** and **4b**: (reaction time 0.5 h), yields 70%; mp >300 °C (from methanol); IR: 3285 (NH), 1705

(CO) cm^{-1}; ^1H NMR: δ 2.17 (s, 3H, CH_3), 2.23 (s, 3H, CH_3), 2.52 (t, 2H, $J = 6.0$ Hz, H-3), 2.58 (s, 3H, CH_3), 4.18 (t, 2H, $J = 6.0$ Hz, H-4), 11.71 (s, 1H, NH); ^{13}C NMR: δ 10.0 (q, CH_3), 10.5 (q, CH_3), 14.7 (q, CH_3), 29.4 (t, C-3), 43.2 (t, C-4), 103.4 (s, C-10a), 108.8 (s, C-10), 128.1 (s, C-9), 145.3 (s, C-7a), 153.4 (s, C-10b), 153.9 (s, C-6), 173.7 (s, C-2). Anal. Calcd for $C_{12}H_{14}N_4OS$: C, 54.94; H, 5.38; N, 21.36; S, 6.10. Found: C, 54.88; H, 5.48; N, 21.50, S, 6.05.

4.12. 10-Methyl-6-(methylsulfanyl)-9-phenyl-3,4-dihydropyrimido[1,2-*c*]pyrrolo[3,2-*e*]pyrimidin-2(8*H*)-one 9b

From **5b** and **4b**: (reaction time 0.5 h), yields 60%; mp >300 °C (from methanol); IR: 3280 (NH), 1710 (CO) cm^{-1}; ^1H NMR: δ 2.53 (s, 3H, CH_3), 2.56 (t, 2H, $J = 6.1$ Hz, H-3), 2.65 (s, 3H, CH_3), 4.23 (t, 2H, $J = 6.1$ Hz, H-4), 7.33–7.62 (m, 5H, Ph), 12.16 (s, 1H, NH); ^{13}C NMR: δ 11.5 (q, CH_3), 14.8 (q, CH_3), 29.3, (t, C-3), 43.3 (t, C-4), 104.1 (s, C-10a), 111.0 (s, C-10), 127.1 (d, C-4′), 127.5 (d, C-2′ and C-6′), 128.6 (d, C-3′ and C-5′), 130.8 (s, C-9), 131.5 (s, C-1′), 146.2 (s, C-7a), 154.4 (s, C-10b), 155.1 (s, C-6), 173.7 (s, C-2). Anal. Calcd for $C_{17}H_{16}N_4OS$: C, 62.94; H, 4.97; N, 17.27; S, 9.88. Found: C, 62.91; H, 4.91; N, 17.37; S, 9.90.

4.13. 5-(Methylsulfanyl)-3,8,9-trimethyl-3*H*-pyrrolo-[3,2-*e*][1,2,4]triazolo[1,5-*c*]pyrimidin-2(7*H*)-one 10a

From **5a** and **4c**: (reaction time 0.5 h), yields 65%; mp >300 °C (from methanol); IR: 3305 (NH), 1705 (CO) cm^{-1}; ^1H NMR: δ 2.23 (s, 6H, $2 \times CH_3$), 2.69 (s, 3H, CH_3), 3.52 (s, 3H, CH_3), 11.94 (s, 1H, NH); ^{13}C NMR: δ 9.1 (q, CH_3), 10.5 (q, CH_3), 14.0 (q, CH_3), 37.8 (q, CH_3), 100.1 (s, C-9a), 107.0 (s, C-9), 129.4 (s, C-8), 143.5 (s, C-6a), 144.6 (s, C-9b), 153.9 (s, C-5), 167.4 (s, C-2). Anal. Calcd for $C_{11}H_{13}N_5OS$: C, 50.18; H, 4.98; N, 26.60; S, 12.18. Found: C, 50.28; H, 4.87; N, 26.49, S, 12.23.

4.14. 3,9-Dimethyl-5-(methylsulfanyl)-8-phenyl-3*H*-pyrrolo[3,2-*e*][1,2,4]triazolo[1,5-*c*]pyrimidin-2(7*H*)-one 10b

From **5b** and **4c**: (reaction time 0.5 h), yields 60%; mp >300 °C (from methanol); IR: 3330 (NH), 1731 (CO) cm^{-1}; ^1H NMR: δ 2.52 (s, 3H, CH_3), 2.75 (s, 3H, CH_3), 3.56 (s, 3H, CH_3) 7.33–7.67 (m, 5H, Ph), 12.46 (s, 1H, NH); ^{13}C NMR: δ 10.9 (q, CH_3), 14.2 (q, CH_3), 37.9 (q, CH_3), 101.0 (s, C-9a), 108.9 (s, C-9), 127.3 (s, C-8), 127.4 (d, C-4′), 127.5 (d, C-2′ and C-6′), 128.7 (d, C-3′ and C-5′), 131.3 (s, C-1′), 131.8 (s, C-6a), 144.4 (s, C-9b), 154.5 (s, C-5), 167.4 (s, C-2). Anal. Calcd for $C_{16}H_{15}N_5OS$: C, 59.06; H, 4.65; N, 21.52; S, 9.85. Found: C, 59.23; H, 4.66; N, 21.60, S, 9.94.

4.15. 5-(Methylsulfanyl)-3,8,9-trimethyl-3*H*-imidazo[1,2-*c*]pyrrolo[3,2-*e*]pyrimidin-2(7*H*)-one 11a

From **5a** and **4d**: (reaction time 1 h), the residue was purified by column chromatography using DCM–MeOH 95:5 as eluant: yields 60%; mp > 300 °C; IR: 3430 (NH), 1701 (CO) cm^{-1}; ^1H NMR: δ 1.55 (d, 3H, $J = 6.9$ Hz, CH_3), 2.20 (s, 6H, $2 \times CH_3$), 2.63 (s, 3H, CH_3), 4.31 (q, 1H, $J = 6.9$ Hz, H-3), 11.95 (s, 1H, NH); ^{13}C NMR: δ 9.2 (q, CH_3), 10.4 (q, CH_3), 13.6 (q, CH_3), 15.7 (q, CH_3), 56.4 (d, C-3), 100.7 (s, C-9a), 108.0 (s, C-9), 128.9 (s, C-8), 146.4 (s, C-6a), 151.1 (s, C-9b), 164.1 (s, C-5), 186.6 (s, C-2). Anal. Calcd for $C_{12}H_{14}N_4OS$: C, 54.94; H, 5.38; N, 21.36; S, 12.22. Found: C, 55.05; H, 5.44; N, 21.39; S, 12.18.

4.16. 3,9-Dimethyl-5-(methylsulfanyl)-8-phenyl-3*H*-imidazo[1,2-*c*]pyrrolo[3,2-*e*]pyrimidin-2(7*H*)-one 11b

From **5b** and **4d**: (reaction time 1 h), yields 43%; mp >300 °C (from toluene); IR: 3446 (NH), 1704 (CO) cm^{-1}; ^1H NMR: δ 1.58 (d, 3H, $J = 7.2$ Hz, CH_3), 2.49 (s, 3H, CH_3), 2.68 (s, 3H, CH_3), 4.36 (q, 1H, $J = 7.2$ Hz, H-3), 7.37 (dt, 1H, $J = 7.2$ and 0.8 Hz, H-4′), 7.48 (dt, 2H, $J = 7.2$ and 0.8 Hz, H-3′ and H-5′), 7.63 (dd, 2H, $J = 7.2$ and 0.8 Hz, H-2′ and H-6′), 12.44 (s, 1H, NH); ^{13}C NMR: δ 11.0 (q, CH_3), 13.8 (q, CH_3), 15.7 (q, CH_3), 56.5 (d, C-3), 101.8 (s, C-9a), 110.0 (s, C-9), 127.3 (d, C-4′, C-2′ and C-6′), 128.7 (d, C-3′ and C-5′), 131.2 (s, C-8), 131.4 (s, C-1′), 147.3 (s, C-6a), 152.6 (s, C-9b), 164.9 (s, C-5), 186.8 (s, C-2). Anal. Calcd for $C_{17}H_{16}N_4OS$: C, 62.94; H, 4.97; N, 17.27; S, 9.88. Found: C, 63.05; H, 4.94; N, 17.25; S, 9.75.

4.17. 6-(Methylsulfanyl)-4,9,10-trimethyl-3,4-dihydropyrimido[1,2-*c*]pyrrolo[3,2-*e*]pyrimidin-2(8*H*)-one 12a

From **5a** and **4e**: (reaction time 0.5 h), the residue was purified by column chromatography using DCM–MeOH 95:5 as eluant: yields 72%; mp 238 °C; IR: 3150 (broad NH), 1655 (CO) cm^{-1}; ^1H NMR: δ 1.29 (d, 3H, $J = 7.4$ Hz, CH_3), 2.17 (s, 3H, CH_3), 2.18 (s, 3H, CH_3), 2.24–2.29 (m, 1H, H-3), 2.75 (s, 3H, CH_3), 2.83 (dd, 1H, $J = 5.9$ and 1.4 Hz, H-3), 4.82–4.86 (m, 1H, H-4), 11.74 (s, 1H, NH); ^{13}C NMR: δ 9.9 (q, CH_3), 10.4 (q, CH_3), 14.6 (q, CH_3), 17.0 (q, CH_3), 36.0 (t, C-3), 50.5 (d, C-4), 103.5 (s, C-10a), 109.0 (s, C-10), 128.2 (s, C-9), 145.1 (s, C-7a), 152.6 (s, C-10b), 152.7 (s, C-6), 173.1 (s, C-2). Anal. Calcd for $C_{13}H_{16}N_4OS$: C, 56.50; H, 5.84; N, 20.27; S, 11.60. Found: C, 56.47; H, 5.93; N, 20.18; S, 11.75.

4.18. 4,10-Dimethyl-6-(methylsulfanyl)-9-phenyl-3,4-dihydropyrimido[1,2-*c*]pyrrolo[3,2-*e*]pyrimidin-2(8*H*)-one 12b

From **5b** and **4e**: (reaction time 0.5 h), the residue was purified by column chromatography using DCM–MeOH 95:5 as eluant: yields 56%; mp 244 °C; IR: 3411 (NH), 1652 (CO) cm^{-1}; ^1H NMR: δ 1.32 (d, 3H, $J = 7.4$ Hz, CH_3), 2.34–2.40 (m, 1H, H-3), 2.54 (s, 3H, CH_3), 2.66 (s, 3H, CH_3), 2.86–2.93 (m, 1H, H-3), 4.80–4.93 (m, 1H, H-4), 7.35 (dt, 1H, $J = 8.8$ and 1.5 Hz, H-4′), 7.47 (dt, 2H, $J = 8.8$ and 1.5 Hz, H-3′ and H-5′), 7.60 (dd, 2H, $J = 8.8$ and 1.5 Hz, H-2′ and H-6′), 12.24 (s, 1H, NH); ^{13}C NMR: δ 11.5 (q, CH_3), 14.7 (q, CH_3), 17.0 (q, CH_3), 35.9 (t, C-3), 50.6 (d, C-4), 104.2 (s, C-10a), 111.1 (s, C-10), 127.1 (d, C-4′), 127.5 (d, C-2′ and C-6′), 128.5 (d, C-3′ and C-5′), 131.0 (s, C-9), 131.5 (s, C-1′), 146.0 (s, C-7a), 153.2 (s, C-10b), 154.1 (s, C-6), 173.2 (s, C-2). Anal. Calcd for $C_{18}H_{18}N_4OS$: C, 63.88; H, 5.36; N, 16.56; S, 9.47. Found: C, 63.92; H, 5.44; N, 26.49; S, 9.55.

4.19. 7,9-Diphenyl-5-(methylsulfanyl)-3*H*-imidazo[1,2-*c*]pyrrolo[3,4-*e*]pyrimidin-2(8*H*)-one 14a

From **13a** and **4a**: (reaction time 4 h), yields 35%; mp >30 °C (methanol); IR: 3232 (NH), 1749 (CO) cm^{-1}; ^1H NMR: δ 2.68 (s, 3H, CH_3), 4.33 (s, 2H, H-3), 7.21–7.53 (m, 6H, H-4′, H-4″, H-2′, H-6′, H-2″ and H-6″, 8.14 (dd, 2H, $J = 8.8$ and 1.5 Hz, H-3′ and H-5′), 8.30 (dd, 2H, $J = 7.4$ and 1.5 Hz, H-3″ and H-5″, 12.68 (s, 1H, NH); ^{13}C NMR: δ 13.6 (q, CH_3), 49.3 (t, C-3), 100.2 (s, C-9a), 121.7 (s, C-9), 125.5 (d, C-2′ and C-6′), 126.2 (d, C-4′), 127.9 (d, C-3′ and C-5′), 128.0 (d, C-4″), 128.4 (d, C-2″ and C-6″), 129.2 (d, C-3″ and C-5″), 130.0 (s, C-7), 130.6 (s, C-1′), 130.8 (s, C-1″), 133.8 (s, C-6a), 148.6 (s, C-9b), 167.3 (s, C-5), 184.3 (s, C-2). Anal. Calcd for $C_{21}H_{16}N_4OS$: C, 67.72; H, 4.33; N, 15.04; S, 8.61. Found: C, 67.79; H, 4.40; N, 15.09; S, 8.68.

4.20. 7,9-Diphenyl-8-methyl-5-(methylsulfanyl)-3*H*-imidazo[1,2-*c*]pyrrolo[3,4-*e*]pyrimidin-2(8*H*)-one 14b

From **13b** and **4a**: (reaction time 4 h), yields 40%; mp >300 °C (methanol); IR: 1724 (CO) cm^{-1}; ^1H NMR: δ 1.91 (s, 3H, CH_3), 3.72 (s, 3H, CH_3), 4.29 (s, H-3), 7.40–7.77 (m, 10H, 2 × Ph); ^{13}C NMR: δ 13.2 (q, CH_3), 35.4 (q, CH_3), 49.3 (t, C-3), 99.9, (s, C-9a), 124.8 (s, C-9), 127.4 (d, C-4′), 127.9 (d, C-2′ and C-6′), 128.4 (d, C-3′ and C-5′), 128.6 (d, C-4″, 129.1 (s, C-1′), 129.5 (s, C-1″, 129.7 (d, C-2″and C-6″, 131.3 (d, C-3″ and C-5″, 131.6 (s, C-7), 132.7 (s, C-6a), 148.5 (s, C-9b), 166.7 (s, C-5), 184.6 (s, C-2). Anal. Calcd for $C_{22}H_{18}N_4OS$: C, 68.37; H, 4.69; N, 14.50; S, 8.30. Found: C, 68.29; H, 4.72; N, 14.46; S, 8.28.

4.21. 8,10-Diphenyl-6-(methylsulfanyl)-3,4-dihydropyrimido[1,2-*c*]pyrrolo[3,4-*e*]pyrimidin-2(9*H*)-one 15a

From **13a** and **4b**: (reaction time 5 h), yields 40%; mp >300 °C (methanol); IR: 3411 (NH), 1665 (CO) cm^{-1}; ^1H NMR: δ 2.50 (s, 3H, CH_3), 2.59 (d, 2H, $J = 7.3$ Hz, H-3), 4.21 (d, 2H, $J = 7.3$ Hz, H-4), 7.24–7.49 (m, 6H, H-4′, H-4″, H-3′, H-5′, H-3″ and H-5″), 7.86 (dd, 2H, $J = 7.3$ and 1.4 Hz, H-2′ and H-6′), 8.27 (d, 2H, $J = 7.3$ Hz, H-2″ and H-6″), 12.53 (s, 1H, NH); ^{13}C NMR: δ 15.0 (q, CH_3), 29.5 (t, C-3), 42.7 (t, C-4), 120.2 (s, C-10a), 125.1 (d, C-2′ and C-6′), 126.0 (d, C-4′), 126.2 (s, C-10), 127.3 (d, C-3′ and C-5′), 127.7 (s, C-1′), 127.8 (d, C-4″), 128.1 (s, C-1″), 128.5 (d, C-2″ and C-6″), 129.8 (s, C-8), 130.5 (d, C-3″ and C-5″), 130.6 (s, C-7a), 130.9 (s, C-10b), 152.2 (s, C-6), 174.7 (s, C-2). Anal. Calcd for $C_{22}H_{18}N_4OS$: C, 68.37; H, 4.69; N, 14.50; S, 8.30. Found: C, 68.42; H, 4.71; N, 14.48; S, 8.38.

4.22. 7,9-Diphenyl-3-methyl-5-(methylsulfanyl)-3*H*-pyrrolo[3,4-*e*][1,2,4]triazolo[1,5-*c*]pyrimidin-2(8*H*)-one 16a

From **13a** and **4c**: (reaction time 8 h), yields 30%; mp >300 °C (methanol); IR: 3410 (NH), 1712 (CO) cm^{-1}; ^1H NMR: δ 2.83 (s, 3H, CH_3), 3.64 (s, 3H, CH_3), 7.51–7.65 (m, 6H, H-4′, H-4″, H-3′, H-5′, H-3″ and H-5″), 8.25 (d, 2H, $J = 7.3$ Hz, H-2′ and H-6′), 8.35 (d, 2H, $J = 7.3$ Hz, H-2″ and H-6″), 12.65 (s, 1H, NH); ^{13}C NMR: δ 14.4 (q, CH_3), 38.2 (q, CH_3), 99.6 (s, C-9a), 122.2 (s, C-9), 125.7 (d, C-4′), 126.7 (s, C-6a), 126.8 (d, C-2′ and C-6′), 128.3 (d, C-4″), 128.4 (d, C-3′ and C-5′), 128.7 (d, C-2″ and C-6″), 129.0 (s, C-1′), 129.3 (d, C-3″ and C-5″), 129.6 (s, C-1″), 136.6 (s, C-7), 145.1 (s, C-9b), 155.8 (s, C-5), 166.3 (s, C-2). Anal. Calcd for $C_{21}H_{17}N_5OS$: C, 65.10; H, 4.42; N, 18.08; S, 8.27. Found: C, 65.22; H, 4.51; N, 18.38; S, 8.38.

4.23. 3-Amino-2,4-dicyano-5-phenylpyrrole 19

Benzylidenemalonitrile **17** (154 g, 1 mol) and pyridinium chloride (0.7 g, 6 mmol) were heated at 160 °C for 3 h in a three-neck round bottom flask under a gaseous chloride stream. The mixture was cooled to room temperature and the crude product **18** was quickly recrystallized from propanol avoiding overboiling. To a mixture of **18** (19 g, 0.1 mol) and aminoacetonitrile bisulfate (15.5 g, 0.1 mol) in absolute ethanol (80 mL) NEt_3 (0.15 mol) was added dropwise and the mixture was stirred at room temperature for 30 min. Then NEt_3 (0.5 mol) was added and the reaction was heated under reflux for 1.5 h. After cooling it was added to water/ice and neutralized with HCl 4 N to pH 6–7. The precipitate was filtered off and purified by column chromatography using DCM–MeOH 9:1 as eluant. Recrystallization from CH_3CN gave **19**, yield 41%, mp 300 °C [lit.[15] 295 °C]; IR: 3454 and 3359 (NH_2), 3197 (NH), 2212 (CN), 2210 (CN) cm^{-1}; ^1H NMR: δ 5.93 (s, 2H, NH_2), 7.43–7.58 (m, 3H, H-4′, H-3′ and H-5′), 7.76 (dd, 2H, $J = 7.3$ and 1.5 Hz, H-2′ and H-6′), 12.34 (s, 1H, NH); ^{13}C NMR: δ 79.8 (s, C-4), 84.8 (s, C-2), 114.2 (s, CN), 115.4 (s, CN), 126.1 (d, C-2′ and C-6′), 128.6 (s, C-1′), 129.1 (d, C-3′ and C-5′), 129.6 (d, C-4′), 139.6 (s, C-3), 148.3 (s, C-5).

4.24. Reaction of 3-amino-2,4-dicyano-5-phenylpyrrole and BMMA

The mixture of **19** (3.27 mmol) and BMMA **4a** (6.54 mmol) in AcOH (10 mL) was stirred under reflux for 18 h. After cooling, the reaction mixture was added to ice/water. The solid was filtered off and purified by column chromatography using DCM–MeOH 98:2. The first compound to be eluted was *5-(methylsulfanyl)-8-oxo-2-phenyl-7,8-dihydro-1H-imidazo[1,2-c]-pyrrolo[2,3-e]pyrimidine-3-carbonitrile* (**23**), yield 40%, mp >300 °C; IR: 3375 (NH), 2229 (CN), 1685 (CO) cm^{-1}; ^1H NMR: δ 2.71 (s, 3H, CH_3), 4.43 (s, 2H, H-7), 7.59–7.62 (m, 3H, H-3′, H-4′ and H-5′), 7.98–8.02 (m, 2H, H-2′ and H-6′); ^{13}C NMR: δ 13.63 (q, CH_3), 50.48 (t, C-7), 84.49 (s, C-3), 111.70 (s, C-3a), 114.72 (s, CN), 127.53 (d, C-2′), 127.53 (d, C-6′), 128.22 (s, C-1′), 129.25 (d, C-3′), 129.25 (d, C-5′), 130.86 (d, C-4′), 147.30 (s, C-2), 148.27 (s, C-9b), 153.49 (s, C-9a), 159.10 (s, C-5), 182.98 (s, C-8). Anal. Calcd for $C_{16}H_{11}N_5OS$: C, 59.80; H, 3.45; N, 21.79; S, 9.98. Found: C, 59.77; H, 3.50; N, 21.83; S, 9.95.

Further elution gave *5-(methylsulfanyl)-2-oxo-9-phenyl-2,8-dihydro-3H-imidazo[1,2-c]pyrrolo[3,4-e]pyrimidine-7-carbonitrile* (**21**), yield 10%, mp 235 °C; IR: 3467 (NH),

2227 (CN), 1655 (CO) cm^{-1}; ^1H NMR: δ 2.09 (s, 3H, CH_3), 3.39 (s, 2H, H-3), 7.22–7.80 (m, 5H, Ph), 12.40 (s, 1H, NH); ^{13}C NMR: δ 22.88 (q, CH_3), 54.85 (t, C-3), 89.65 (s, C-7), 115.40 (s, CN), 119.98 (s, C-6a), 126.78 (d, C-2′), 126.78 (d, C-6′), 126.89 (s, C-9a), 128.85 (s, C-1′), 128.93 (d, C-3′), 128.93 (d, C-5′), 129.26 (d, C-4′), 138.02 (s, C-9), 146.48 (s, C-9b), 160.64 (s, C-5), 169.04 (s, C-2). Anal. Calcd for $C_{16}H_{11}N_5OS$: C, 59.80; H, 3.45; N, 21.79; S, 9.98. Found: C, 59.85; H, 3.42; N, 21.73; S, 10.04.

References and notes

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16. Semi-empirical molecular orbital calculations were run on an Indigo-2 Silicon Graphics work station by using the Vamp (V 6.5) software, supplied by Oxford Molecular—Accelrys. The structures of the molecules were fully optimized in vacuo and in DMSO by SCF calculation with PM3 method according to Stewart, J. J. P. *J. Comput. Chem.* **1989**, *10*, 209–221. A prediction of the ^{13}C NMR chemical shifts by the neural-net technique was obtained according to Clark, T.; Ruahut, G.; Breindl, A. *J. Mol. Mod.* **1955**, *1*, 22. Tables with full data are available as supplementary material.
17. In the protocol, each cell line is inoculated and preincubated on a microtiter plate. Test agents are then added at a single concentration and the culture incubated for 48 h. End-point determinations are made with alamar blue (Gray, G. D.; Wickstrom, E. *Biotechniques* **1996**, *21*, 780) Results for each test agent are reported as the percent of growth of the treated cells when compared to the untreated control cells. Compounds which reduce the growth of any one of the cell lines to approximately 32% or less (negative numbers indicate cell kill) are passed on for evaluation in the full panel of 60 cell lines over a 5-log dose range.
18. Test compounds, dissolved in $(CH_3)_2SO$ at an initial concentration of 200 mM and serially diluted in culture medium, were incubated in the presence of LoVo cells for 72 h at 37 °C. The number of viable cells was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method (Denizot, F.; Lang, R. *J. Immunol. Methods* **1986**, *89*, 271). Tumor cell growth at each drug concentration was expressed as percentage of untreated controls and the concentration resulting in 50% (IC_{50}) growth inhibition was determined by linear regression analysis. For doxorubicin, used as reference drug, IC_{50} = 2.5 μM.
19. The calculations were performed with the software TSAR (V 3.2), supplied by Oxford Molecular—Accelrys, running on an Indigo II Silicon Graphics work station.
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