Supporting Information:

Truncated Nucleosides as A_3 Adenosine Receptor Ligands: Combination of 2-Arylethynyl and Bicyclohexane Substitutions

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Abbreviations: ADA, adenosine deaminase; AR, adenosine receptor; cyclic AMP, adenosine 3′,5′-cyclic phosphate; CHO, Chinese hamster ovary; Cl-IB-MECA, 2-chloro-N^6-(3-iodobenzyl)-5′-*N*-methylcarboxamidoadenosine; DMEM, Dulbecco's modified Eagle's medium; EDTA, ethylenediaminetetraacetic acid; GPCR, G protein-coupled receptor; HEK, human embryonic kidney; I-AB-MECA, 2-[*p*-(2-carboxyethyl)phenyl-ethylamino]-5′-*N*-ethylcarboxamidoadenosine; NECA, 5′-*N*-ethylcarboxamidoadenosine; DMF, *N*,*N*-dimethylformamide; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HRMS, high resolution mass spectroscopy; TEA, triethylamine; TLC, thin layer chromatography.

Chemical synthesis

Chemistry

General Methods

All reagents and solvents (regular and anhydrous) were of analytical grade and obtained from commercial suppliers and used without further purification. The substituted phenyl alkynes to produce protected nucleoside intermediates **31**-**48** were obtained from Sigma-Aldrich (St. Louis, MO). Reactions were conducted under an atmosphere of nitrogen whenever anhydrous solvents were used. All reactions were monitored by thin-layer chromatography (TLC) using silica gel coated plates with a fluorescence indicator which were visualized: (a) under UV light, (b) by dipping in a mixture of anisaldehyde (2.5 mL)/ conc. H_2SO_4 (5 mL)/methanol (425 mL) or (c) by dipping the plate in a solution of ninhydrin (0.3 g in 100 mL EtOH, containing AcOH, 1.3 mL) followed by heating. Silica gel column chromatography was performed with silica gel (SiO_2, 200-400 mesh, 60 Å) using moderate air pressure. Evaporation of solvents was carried out under reduced pressure at a temperature below 50 ºC. After column chromatography, appropriate fractions were pooled, evaporated, and dried at high vacuum for at least 12 h to give the desired products in high purity. ^{1}H NMR spectra were recorded with a Bruker 400 MHz NMR spectrometer. Chemical shifts are reported in parts per million (ppm) relative to tetramethylsilane or using deuterated solvent as the internal standard (δH: $CDCl_3$ 7.26 ppm). ESI - High Resolution Mass Spectroscopic (HRMS) measurements were performed on a proteomics optimized Q-TOF-2 (Micromass-Waters) using external calibration with polyalanine. Observed mass accuracies are those expected on the basis of known performance of the instrument as well as the trends in masses of standard compounds observed at intervals during the series of measurements. Reported masses are observed masses uncorrected for this time-dependent drift in mass accuracy. The purity of the final nucleoside derivatives was determined using a Hewlett–Packard 1100 HPLC equipped with a Zorbax Eclipse 5 mm XDB-C18 analytical column (250 X 4.6 mm; Agilent Technologies Inc, Palo Alto, CA), using a linear gradient solvent system: 5 mM TBAP (tetrabutylammonium dihydrogenphosphate)-CH_3CN from 80:20 to 40:60 in 20 min with a flow rate of 1 mL/min. Peaks were detected by UV absorption (254 nm) using a diode array detector.

All derivatives tested for biological activity were shown to be at least 95% pure using this analytical HPLC system. Compounds **23** and **24** were prepared as reported.[1,2]

(*1R,2R,3S,4R,5S*)-4-(6-(Methylamino)-2-(phenylethynyl)-*9H*-purin-9-yl) bicyclo[3.1.0]hexane-2,3-diol (4)

$PdCl_2(PPh_3)_2$ (4.0 mg, 0.005 mmol), CuI (1.0 mg, 0.003 mmol), phenylacetylene (19 μL, 0.17 mmol) and triethylamine (40 μL, 0.28 mmol) were added to a solution of compound **25** (12.35 mg, 0.028 mmol) in anhydrous DMF (0.8 mL), and the mixture was stirred at room temperature overnight. Solvent was evaporated under vacuum, and the residue was purified roughly on flash silica gel column chromatography, to yield intermediate **31**. The resulting compound was dissolved in methanol (1.5 mL)-dichloromethane (0.5 mL), 50% trifluoromethane sulfonic acid (2 mL) was added to the reaction mixture, and it was stirred at room temperature overnight. Solvent was evaporated under vacuum, and the residue was purified on flash silica gel column chromatography (CH_2Cl_2:MeOH = 35:1) to give compound **4** (8 mg, 77%) as a syrup. ^{1}H NMR (CD_3OD, 400 MHz) δ 8.23 (s, 1H), 7.67-7.65 (m, 2H), 7.47-7.43 (m, 3H), 4.88 (s, 1H), 4.70 (t, *J* = 6.0 Hz, 1H), 3.91 (d, *J* = 6.8 Hz, 1H), 3.17 (br s, 3H), 2.04-1.99 (m, 1H), 1.75-1.70 (m, 1H), 1.40-1.36 (m, 1H), 0.82-0.76 (m, 1H). HRMS calculated for $C_{20}H_{20}N_5O_2$ (M + H) $^+$: 362.1617; found 362.1613.

(*1R,2R,3S,4R,5S*)-4-(2-((2-Chloro-phenyl)ethynyl)-6-(methylamino)-*9H*-purin-9-yl) bicyclo[3.1.0]hexane-2,3-diol (5)

Compound **5** (74%) was prepared from compound **25** following the same method for compound **4**. ^{1}H NMR (CD_3OD, 400 MHz) δ 8.23 (s, 1H), 7.72 (dd, J_1 = 1.6 Hz, J_2 = 6.0 Hz, 1H), 7.55-7.53 (m, 1H), 7.46-7.36 (m, 2H), 4.96 (s, 1H), 4.73 (t, *J* = 6.0 Hz, 1H), 3.91 (d, *J* = 6.4 Hz, 1H), 3.17 (br s, 3H), 2.04-1.99 (m, 1H), 1.74-1.69 (m, 1H), 1.38-1.35 (m, 1H), 0.81-0.76 (m, 1H). HRMS calculated for $C_{20}H_{19}ClN_5O_2$ (M + H) $^+$: 396.1227; found 396.1222.

(*1R,2R,3S,4R,5S*)-4-(6-(Methoxyamino)-2-(phenylethynyl)-*9H*-purin-9-yl) bicyclo[3.1.0]hexane-2,3-diol (6)

Compound **6** (54%) was prepared from compound **26** following the same method for compound **4**. ^{1}H NMR (CD_3OD, 400 MHz) δ 8.31 (s, 1H), 7.67-7.66 (m, 2H), 7.47-7.45 (m, 3H), 4.72 (t, *J* = 5.6 Hz, 1H), 3.90 (m, 4H), 2.07-1.98 (m, 1H), 1.78-1.69 (m, 1H),

1.41-1.36 (m, 1H), 0.80-0.77 (m, 1H). HRMS calculated for $C_{20}H_{20}N_5O_3$ $(M + H)^+$: 378.1566; found 378.1578.

(*1R,2R,3S,4R,5S*)-4-(2-((2-Chloro-phenyl)ethynyl)-6-(methoxyamino)-*9H*-purin-9-yl) bicyclo[3.1.0]hexane-2,3-diol (7)

Compound **7** (63%) was prepared from compound **26** following the same method for compound **4**. ^{1}H NMR (CD_3OD, 400 MHz) δ 8.32 (s, 1H), 7.75 (d, *J* = 7.6 Hz, 1H), 7.54 (d, *J* = 8.0 Hz, 1H), 7.50-7.37 (m, 2H), 4.75 (t, *J* = 6.4 Hz, 1H), 3.94 (d, *J* = 6.0 Hz, 1H), 3.91 (s. 3H), 2.08-1.97 (m, 1H), 1.76-1.68 (m, 1H), 1.38-1.34 (m, 1H), 0.82-0.76 (m, 1H). HRMS calculated for $C_{20}H_{19}ClN_5O_3$ $(M + H)^+$: 412.1176; found 412.1166.

(*1R,2R,3S,4R,5S*)-4-(6-(Ethylamino)-2-(phenylethynyl)-*9H*-purin-9-yl) bicyclo[3.1.0]hexane-2,3-diol (8)

Compound **8** (63%) was prepared from compound **27** following the same method for compound **4**. ^{1}H NMR (CD_3OD, 400 MHz) δ 8.23 (s, 1H), 7.67-7.64 (m, 2H), 7.46-7.44 (m, 3H), 4.70 (t, *J* = 5.6 Hz, 1H), 4.59 (br s, 2H), 3.91 (d, *J* = 6.4 Hz, 1H), 2.04-1.99 (m, 1H), 1.75-1.71 (m, 1H), 1.39-1.31 (m, 4H), 0.82-0.77 (m, 1H). HRMS calculated for $C_{21}H_{22}N_5O_2$ $(M + H)^+$: 376.1774; found 376.1794.

(*1R,2R,3S,4R,5S*)-4-(2-((2-Chloro-phenyl)ethynyl)-6-(ethylamino)-*9H*-purin-9-yl) bicyclo[3.1.0]hexane-2,3-diol (9)

Compound **9** (67%) was prepared from compound **27** following the same method for compound **4**. ^{1}H NMR (CD_3OD, 400 MHz) δ 8.23 (s, 1H), 7.72 (d, *J* = 7.6 Hz, 1H), 7.53 (d, *J* = 7.6 Hz, 1H), 7.45-7.36 (m, 2H), 4.90 (s, 1H), 4.73 (t, *J* = 5.6 Hz, 1H), 4.58 (br s, 2H), 3.91 (d, *J* = 6.4 Hz, 1H), 2.04-1.99 (m, 1H), 1.74-1.69 (m, 1H), 1.38-1.31 (m, 4H), 0.81-0.76 (m, 1H). HRMS calculated for $C_{21}H_{21}ClN_5O_2$ $(M + H)^+$: 410.1384; found 410.1383.

(*1R,2R,3S,4R,5S*)-4-(6-(3-Chloro-benzylamino)-2-(phenylethynyl)-*9H*-purin-9-yl) bicyclo[3.1.0]hexane-2,3-diol (10)

Compound **10** (72%) was prepared from compound **28** following the same method for compound **4**. ^{1}H NMR (CD_3OD, 400 MHz) δ 8.25 (s, 1H), 7.65-7.63 (m, 2H), 7.47-7.43 (m, 4H), 7.38-7.26 (m, 3H), 4.72 (t, *J* = 5.6 Hz, 1H), 3.92 (d, *J* = 6.4 Hz, 1H), 2.05-2.00 (m, 1H), 1.78-1.69 (m, 1H), 1.42-1.36 (m, 1H), 0.81-0.76 (m, 1H). HRMS calculated for $C_{26}H_{23}ClN_5O_2$ $(M + H)^+$: 472.1540; found 472.1540.

(*1R,2R,3S,4R,5S*)-4-(6-(3-Chloro-benzylamino)-2-((3,4-difluorophenyl)ethynyl)-*9H*-purin-9-yl)bicyclo[3.1.0]hexane-2,3-diol (11)

Compound **11** (71%) was prepared from compound **28** following the same method for compound **4**. ^{1}H NMR (CD_3OD, 400 MHz) δ 8.26 (s, 1H), 7.60-7.55 (m, 1H), 7.50-7.45 (m, 2H), 7.39-7.28 (m, 4H), 4.92 (s, 1H), 4.71 (t, *J* = 5.6 Hz, 1H), 3.93 (d, *J* = 6.8 Hz, 1H), 2.04-1.98 (m, 1H), 1.74-1.70 (m, 1H), 1.38-1.35 (m, 1H), 0.82-0.76 (m, 1H). HRMS calculated for $C_{26}H_{21}ClF_2N_5O_2$ $(M + H)^+$: 508.1352; found 508.1347.

(*1R,2R,3S,4R,5S*)-4-(6-(3-Chloro-benzylamino)-2-((2-chlorophenyl)ethynyl)-*9H*-purin-9-yl)bicyclo[3.1.0]hexane-2,3-diol (12)

Compound **12** (82%) was prepared from compound **28** following the same method for compound **4**. ^{1}H NMR (CD_3OD, 400 MHz) δ 8.25 (s, 1H), 7.72-7.63 (m, 1H), 7.58-7.49 (m, 1H), 7.43-7.26 (m, 6H), 4.73 (t, *J* = 5.6 Hz, 1H), 3.93 (d, *J* = 6.8 Hz, 1H), 2.01-1.97 (m, 1H), 1.71-1.69 (m, 1H), 1.36-1.34 (m, 1H), 0.84-0.75 (m, 1H). HRMS calculated for $C_{26}H_{22}Cl_2N_5O_2$ $(M + H)^+$: 506.1151; found 506.1142.

(*1R,2R,3S,4R,5S*)-4-(6-(3-Chloro-benzylamino)-2-((3-chlorophenyl)ethynyl)-*9H*-purin-9-yl)bicyclo[3.1.0]hexane-2,3-diol (13)

Compound **13** (75%) was prepared from compound **28** following the same method for compound **4**. ^{1}H NMR (CD_3OD, 400 MHz) δ 8.26 (s, 1H), 7.68-7.61 (m, 1H), 7.59-7.51 (m, 1H), 7.49-7.41 (m, 3H), 7.38-7.31 (m, 3H), 4.92 (s, 1H), 4.71 (t, *J* = 5.2 Hz, 1H), 3.93 (d, *J* = 6.4 Hz, 1H), 2.04-1.98 (m, 1H), 1.75-1.71 (m, 1H), 1.39-1.36 (m, 1H), 0.82-0.78 (m, 1H). HRMS calculated for $C_{26}H_{22}Cl_2N_5O_2$ $(M + H)^+$: 506.1151; found 506.1131.

(*1R,2R,3S,4R,5S*)-4-(2-(Biphenyl-4-ylethynyl)-6-(3-chlorobenzylamino)-*9H*-purin-9-yl)bicyclo[3.1.0]hexane-2,3-diol (14)

Compound **14** (70%) was prepared from compound **28** following the same method for compound **4**. ^{1}H NMR (CD_3OD, 400 MHz) δ 8.26 (s, 1H), 7.78-7.65 (m, 6H), 7.52-7.44 (m, 3H), 7.43-7.25 (m, 4H), 4.72 (t, *J* = 5.6 Hz, 1H), 3.93 (d, *J* = 6.8 Hz, 1H), 2.06-1.97 (m, 1H), 1.78-1.69 (m, 1H), 1.43-1.37 (m, 1H), 0.85-0.76 (m, 1H). HRMS calculated for $C_{32}H_{27}ClN_5O_2$ $(M + H)^+$: 548.1853; found 548.1849.

(*1R,2R,3S,4R,5S*)-4-(6-(Phenethylamino)-2-(phenylethynyl)-*9H*-purin-9-yl) bicycle [3.1.0]hexane-2,3-diol (15)

Compound **15** (69%) was prepared from compound **29** following the same method for compound **4**. ^{1}H NMR (CD_3OD, 400 MHz) δ 8.22 (s, 1H), 7.67-7.65 (m, 2H), 7.45-7.44 (m, 3H), 7.33-7.27 (m, 4H), 7.21-7.18 (m, 1H), 4.69 (t, *J* = 5.6 Hz, 1H), 4.59 (br s, 2H), 3.90 (d, *J* = 6.4 Hz, 1H), 3.03 (t, *J* = 7.2 Hz, 2H), 2.03-1.99 (m, 1H), 1.74-1.71 (m, 1H), 1.39-1.36 (m, 1H), 0.82-0.76 (m, 1H). HRMS calculated for $C_{27}H_{26}N_5O_2$ (M + H) $^+$: 452.2087; found 452.2070.

(*1R,2R,3S,4R,5S*)-4-(2-((2-Chlorophenyl)ethynyl)-6-(phenethylamino)-*9H*-purin-9-yl)bicyclo[3.1.0]hexane-2,3-diol (16)

Compound **16** (83%) was prepared from compound **29** following the same method for compound **4**. ^{1}H NMR (CD_3OD, 400 MHz) δ 8.24 (s, 1H), 7.73(d, *J* = 6.4 Hz, 1H), 7.56-7.54 (m, 1H), 7.46-7.31 (m, 6H), 7.29-7.19 (m, 1H), 4.72 (t, *J* = 5.6 Hz, 1H), 4.59 (br s, 2H), 3.90 (d, *J* = 6.4 Hz, 1H), 3.03 (t, *J* = 7.2 Hz, 2H), 2.04-1.99 (m, 1H), 1.76-1.68 (m, 1H), 1.40-1.32 (m, 1H), 0.85-0.76 (m, 1H). HRMS calculated for $C_{27}H_{25}ClN_5O_2$ (M + H) $^+$: 486.1697; found 486.1712.

(*1R,2R,3S,4R,5S*)-4-(6-(2,2-diphenylethylamino)-2-(phenylethynyl)-*9H*-purin-9-yl)bicyclo[3.1.0]hexane-2,3-diol (17)

Compound **17** (82%) was prepared from compound **30** following the same method for compound **4**. ^{1}H NMR (CD_3OD, 400 MHz) δ 8.18 (s, 1H), 7.68-7.65 (m, 2H), 7.46-7.43 (m, 3H), 7.42-7.34 (m, 4H), 7.32-7.28 (m, 4H), 7.22-7.18 (m, 2H), 4.93 (s, 1H), 4.67 (t, *J* = 5.6 Hz, 1H), 4.52 (t, *J* = 7.6 Hz, 1H), 4.30 (br s, 2H), 3.89 (d, *J* = 6.4 Hz, 1H), 2.02-1.96 (m, 1H), 1.71-1.69 (m, 1H), 1.38-1.35 (m, 1H), 0.80-0.75 (m, 1H). HRMS calculated for $C_{33}H_{30}N_5O_2$ (M + H) $^+$: 528.2400; found 528.2395.

(*1R,2R,3S,4R,5S*)-4-(2-((3,4-Difluorophenyl)ethynyl)-6-(2,2-diphenylethylamino)-*9H*-purin-9-yl)bicyclo[3.1.0]hexane-2,3-diol (18)

Compound **18** (68%) was prepared from compound **30** following the same method for compound **4**. ^{1}H NMR (CD_3OD, 400 MHz) δ 8.20 (s, 1H), 7.70-7.64 (m, 1H), 7.63-7.56 (m, 1H), 7.53-7.48 (m, 1H), 7.42-7.37 (m, 4H), 7.34-7.27 (m, 4H), 7.25-7.17 (m, 2H), 4.68 (t, *J* = 5.6 Hz, 1H), 4.51 (t, *J* = 8.0 Hz, 1H), 4.29 (br s, 2H), 3.90 (d, *J* = 6.4 Hz, 1H), 2.04-1.96 (m, 1H), 1.75-1.67 (m, 1H), 1.41-1.34 (m, 1H), 0.84-0.74 (m, 1H). HRMS calculated for $C_{33}H_{28}F_2N_5O_2$ (M + H) $^+$: 564.2211; found 564.2211.

(*1R,2R,3S,4R,5S*)-4-(2-((2-Chlorophenyl)ethynyl)-6-(2,2-diphenylethylamino)-*9H*-purin-9-yl)bicyclo[3.1.0]hexane-2,3-diol (19)

Compound **19** (79%) was prepared from compound **30** following the same method for compound **4**. ^{1}H NMR (CD_3OD, 400 MHz) δ 8.19 (s, 1H), 7.68-7.63 (m, 2H), 7.59-7.54 (m, 2H), 7.42-7.78 (m, 4H), 7.35-7.28 (m, 4H), 7.24-7.18 (m, 2H), 4.93 (s, 1H), 4.70 (t, *J* = 5.6 Hz, 1H), 4.57 (t, *J* = 7.6 Hz, 1H), 4.29 (br s, 2H), 3.89 (d, *J* = 6.8 Hz, 1H), 2.02-1.96 (m, 1H), 1.70-1.68 (m, 1H), 1.37-1.34 (m, 1H), 0.80-0.74 (m, 1H). HRMS calculated for $C_{33}H_{29}ClN_5O_2$ $(M + H)^+$: 562.2010; found 562.2000.

(*1R,2R,3S,4R,5S*)-4-(2-((3-Chlorophenyl)ethynyl)-6-(2,2-diphenylethylamino)-*9H*-purin-9-yl)bicyclo[3.1.0]hexane-2,3-diol (20)

Compound **20** (87%) was prepared from compound **30** following the same method for compound **4**. ^{1}H NMR (CD_3OD, 400 MHz) δ 8.19 (s, 1H), 7.72-7.76 (m, 1H), 7.63-7.56 (m, 1H), 7.52-7.43 (m, 2H), 7.41-7.36 (m, 4H), 7.33-7.28 (m, 4H), 7.24-7.17 (m, 2H), 4.68 (t, *J* = 5.6 Hz, 1H), 4.53 (t, *J* = 7.6 Hz, 1H), 4.30 (br s, 2H), 3.89 (d, *J* = 6.8 Hz, 1H), 2.03-1.97 (m, 1H), 1.73-1.67 (m, 1H), 1.39-1.34 (m, 1H), 0.82-0.72 (m, 1H). HRMS calculated for $C_{33}H_{29}ClN_5O_2$ $(M + H)^+$: 562.2010; found 562.2014.

(*1R,2R,3S,4R,5S*)-4-(2-(Biphenyl-4-ylethynyl)-6-(2,2-diphenylethylamino)-*9H*-purin-9-yl)bicyclo[3.1.0]hexane-2,3-diol (21)

Compound **21** (78%) was prepared from compound **30** following the same method for compound **4**. ^{1}H NMR (CD_3OD, 400 MHz) δ 8.19 (s, 1H), 7.77-7.63 (m, 6H), 7.62-7.45 (m, 3H), 7.43-7.36 (m, 4H), 7.34-7.28 (m, 4H), 7.22-7.19 (m, 2H), 4.68 (t, *J* = 5.6 Hz, 1H), 4.52 (t, *J* = 7.6 Hz, 1H), 4.32 (br s, 2H), 3.90 (d, *J* = 6.4 Hz, 1H), 2.03-1.97 (m, 1H), 1.76-1.67 (m, 1H), 1.42-1.35 (m, 1H), 0.84-0.76 (m, 1H). HRMS calculated for $C_{39}H_{34}N_5O_2$ $(M + H)^+$: 604.2713; found 604.2714.

(*1R,2R,3S,4R,5S*)-4-(2-Iodo-6-(methylamino)-*9H*-purin-9-yl)-2′,3′-*O*-(isopropylidene)-bicyclo[3.1.0]hexane (25)

Methylamine hydrochloride (49 mg, 0.72 mmol) and triethylamine (0.28 mL, 1.96 mmol) were added to a solution of compound **24** (62.8 mg, 0.14 mmol) in methanol (2.0 mL) and stirred at room temperature overnight. The reaction mixture was evaporated under vacuum, and the residue was purified on flash column chromatography (hexane:ethyl acetate = 1:1) to give the desired product **25** (51 mg, 83%) as a syrup. ^{1}H NMR (CD_3OD,

400 MHz) δ 8.02 (s, 1H), 5.36 (t, *J* = 6.8 Hz, 1H), 4.96 (s, 1H), 4.70 (d, *J* = 7.2 Hz, 1H), 3.07 (br s, 3H), 2.07-2.01 (m, 1H), 1.72-1.67 (m, 1H), 1.52 (s, 3H), 1.26 (s, 3H), 0.96-0.92 (m, 2H). HRMS calculated for $C_{15}H_{19}IN_5O_2$ $(M + H)^+$: 428.0584; found 428.0600.

(*1R,2R,3S,4R,5S*)-4-(2-Iodo-6-(methoxyamino)-*9H*-purin-9-yl)-2′,3′-*O*-(isopropylidene)-bicyclo[3.1.0]hexane (26)

Compound **26** (65%) was prepared from compound **24** following the same method for compound **25**. ^{1}H NMR (CD_3OD, 400 MHz) δ 8.13 (s, 1H), 5.38 (t, *J* = 6.8 Hz, 1H), 4.98 (s, 1H), 4.73 (d, *J* = 6.8 Hz, 1H), 3.87 (s, 3H), 2.08-2.04 (m, 1H), 1.73-1.69 (m, 1H), 1.52 (s, 3H), 1.26 (s, 3H), 0.94-0.89 (m, 2H). HRMS calculated for $C_{15}H_{19}IN_5O_3$ $(M + H)^+$: 444.0533; found 444.0526.

(*1R,2R,3S,4R,5S*)-4-(2-Iodo-6-(ethylamino)-*9H*-purin-9-yl)-2′,3′-*O*-(isopropylidene)-bicyclo[3.1.0] hexane (27)

Compound **27** (78%) was prepared from compound **24** following the same method for compound **25**. ^{1}H NMR (CD_3OD, 400 MHz) δ 8.03 (s, 1H), 5.36 (t, *J* = 6.8 Hz, 1H), 4.96 (s, 1H), 4.70 (d, *J* = 7.2 Hz, 1H), 4.6 (br s, 2H), 2.07-2.01 (m, 1H), 1.72-1.69 (m, 1H), 1.52 (s, 3H), 1.30-123 (m, 6H), 0.93-0.88 (m, 2H). HRMS calculated for $C_{16}H_{21}IN_5O_2$ $(M + H)^+$: 442.0740; found 442.0740.

(*1R,2R,3S,4R,5S*)-4-(2-Iodo-6-(3-chloro-benzylamino)-*9H*-purin-9-yl)-2′,3′-*O*-(isopropylidene)-bicyclo[3.1.0]hexane (28)

Compound **28** (74%) was prepared from compound **24** following the same method for compound **25**. ^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.63 (s, 1H), 7.37 (s, 1H), 7.27-7.26 (m, 3H), 6.25 (br s, 1H), 5.34 (t, *J* = 5.7 Hz, 1H), 4.94 (s, 2H), 4.64 (d, *J* = 6.9 Hz, 1H), 2.06-2.09 (m, 1H), 1.61-1.69 (m, 2H), 1.54 (s, 3H), 1.26 (s, 3H), 0.88-0.96 (m, 2H). HRMS calculated for $C_{21}H_{22}ClIN_5O_2$ $(M+H)^+$: 538.0497; found 538.0507.

(*1R,2R,3S,4R,5S*)-4-(2-Iodo-6-(2-phenylethylamino)-*9H*-purin-9-yl)-2′,3′-*O*-(isopropylidene)-bicyclo[3.1.0]hexane (29)

Compound **29** (84%) was prepared from compound **24** following the same method for compound **25**. ^{1}H NMR (CD_3OD, 400 MHz) δ 8.02 (s, 1H), 7.28 (s, 4H), 7.19 (m, 1H) 5.36 (t, *J* = 6.4 Hz, 1H), 4.95 (s, 1H), 4.69 (d, *J* = 6.4 Hz, 1H), 3.78 (br s, 2H), 2.97 (t, *J* = 6.0 Hz, 2H), 2.03-1.99 (m, 1H), 1.70-1.69 (m, 1H), 1.51 (s, 3H), 1.25 (s, 3H), 0.93-0.89 (m, 2H). HRMS calculated for $C_{22}H_{25}IN_5O_2$ $(M + H)^+$: 518.1053; found 518.1037.

(*1R,2R,3S,4R,5S*)-4-(2-Iodo-6-(2,2-diphenylethylamino)-*9H*-purin-9-yl)-2′,3′-*O*-(isopropylidene)-bicyclo[3.1.0]hexane (30)

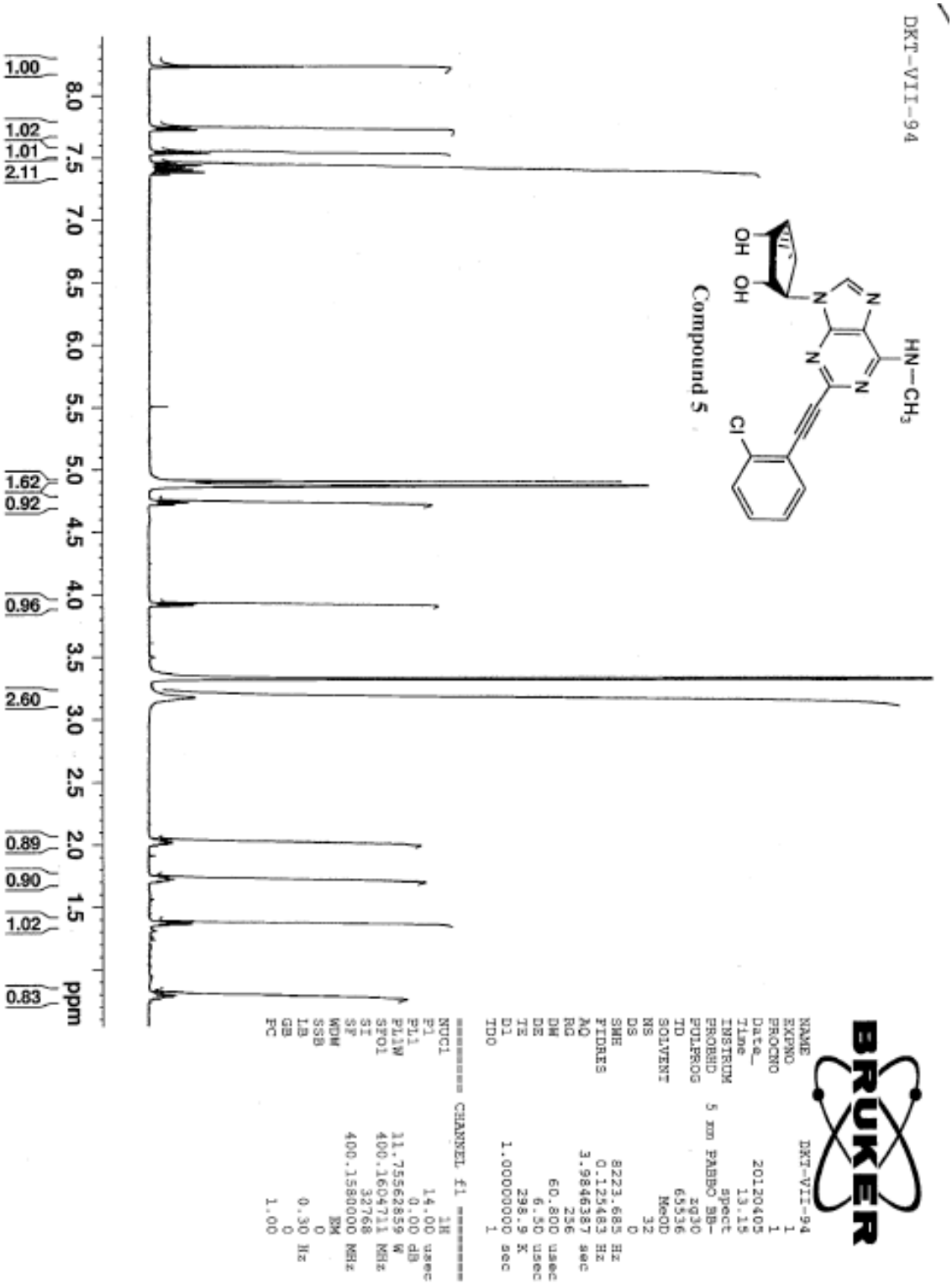
Compound **30** (79%) was prepared from compound **24** following the same method for compound **25**. ^{1}H NMR (CD_3OD, 400 MHz) δ 8.01 (s, 1H), 7.36-7.28 (m, 9H), 7.21-7.17 (m, 1H) 5.34 (t, *J* = 6.4 Hz, 1H), 4.93 (s, 1H), 4.67 (d, *J* = 6.8 Hz, 1H), 4.49 (d, *J* = 7.6 Hz, 1H), 4.17 (d, *J* = 7.6 Hz, 2H), 2.05-1.99 (m, 1H), 1.69-1.65 (m, 1H), 1.51 (s, 3H), 1.24 (s, 3H), 0.93-0.86 (m, 2H). HRMS calculated for $C_{28}H_{29}IN_5O_2$ $(M + H)^+$: 594.1366; found 594.1382.

References:

1. Choi, W. J.; Park, J. G.; Yoo, S. J.; Kim, H. P.; Moon, H. R.; Chun, M. W.; Jung, Y. H.; Jeong, L. S. Syntheses of D- and L-cyclopentenone derivatives using ring-closing metathesis: versatile intermediates for the synthesis of D- and L-carbocyclic nucleosides. *J. Org. Chem.* **2001**, *66*, 6490–6494.

2. Joshi, B.V.; Melman, A.; Mackman, R.L.; Jacobson, K.A. Synthesis of ethyl (1*S*,2*R*,3*S*,4*S*,5*S*)-2,3-*O*-(isopropylidene)-4-hydroxy-bicyclo [3.1.0] hexane-carboxylate from L-ribose: a versatile chiral synthon for preparation of adenosine and P2 receptor ligands. *Nucleosides, Nucleotides, and Nucleic Acids,* **2008**, *27*, 279-291.

Spectroscopic characterization of selected nucleoside derivatives.



Elemental Composition Report Page 1

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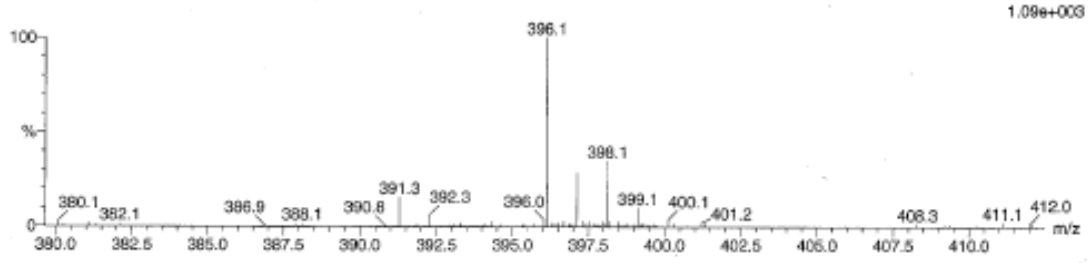

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	396.1214	0.8	2.0	8.5	157.9	C19 H23 N O6 35Cl
	396.1268	-4.6	-11.6	17.5	148.8	C25 H19 N3 35Cl
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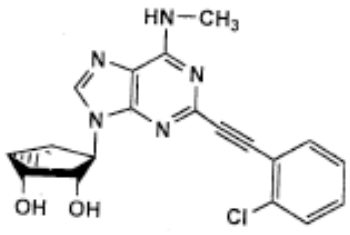
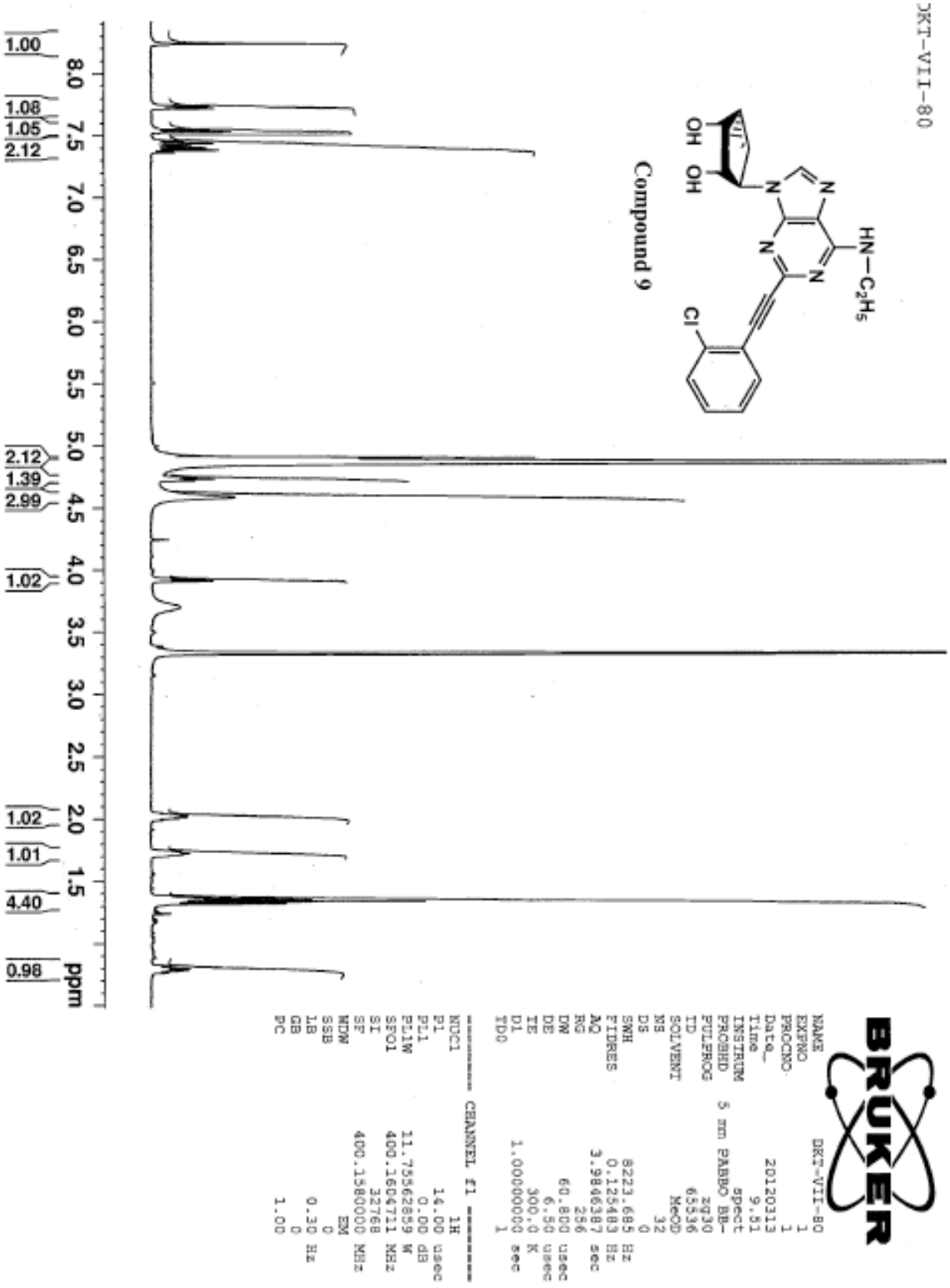


Compound 5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2.99
1.02
1.02
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D1 1.00000000 sec
TD0 1
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SI 32768
SF 400.1580000 MHz
WDW EM
SSB 0
LB 0.30 Hz
GB 0
PC 1.00

Elemental Composition Report **Page 1**

Single Mass Analysis
Tolerance = 9.0 mDa / DBE: min = -1.5, max = 50.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 3

Monoisotopic Mass, Even Electron Ions
226 formula(e) evaluated with 4 results within limits (up to 50 closest results for each mass)
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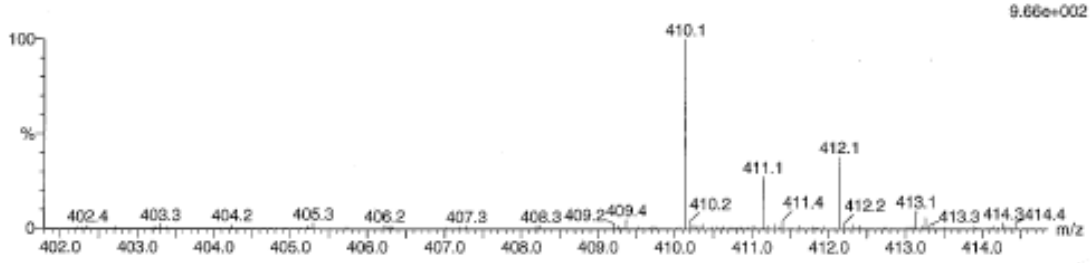
9.66e+002



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	410.1424	-4.1	-10.0	17.5	145.7	C26 H21 N3 35Cl
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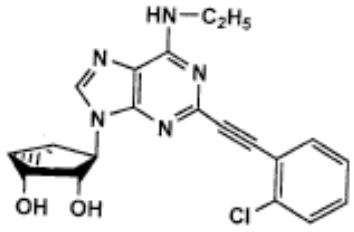
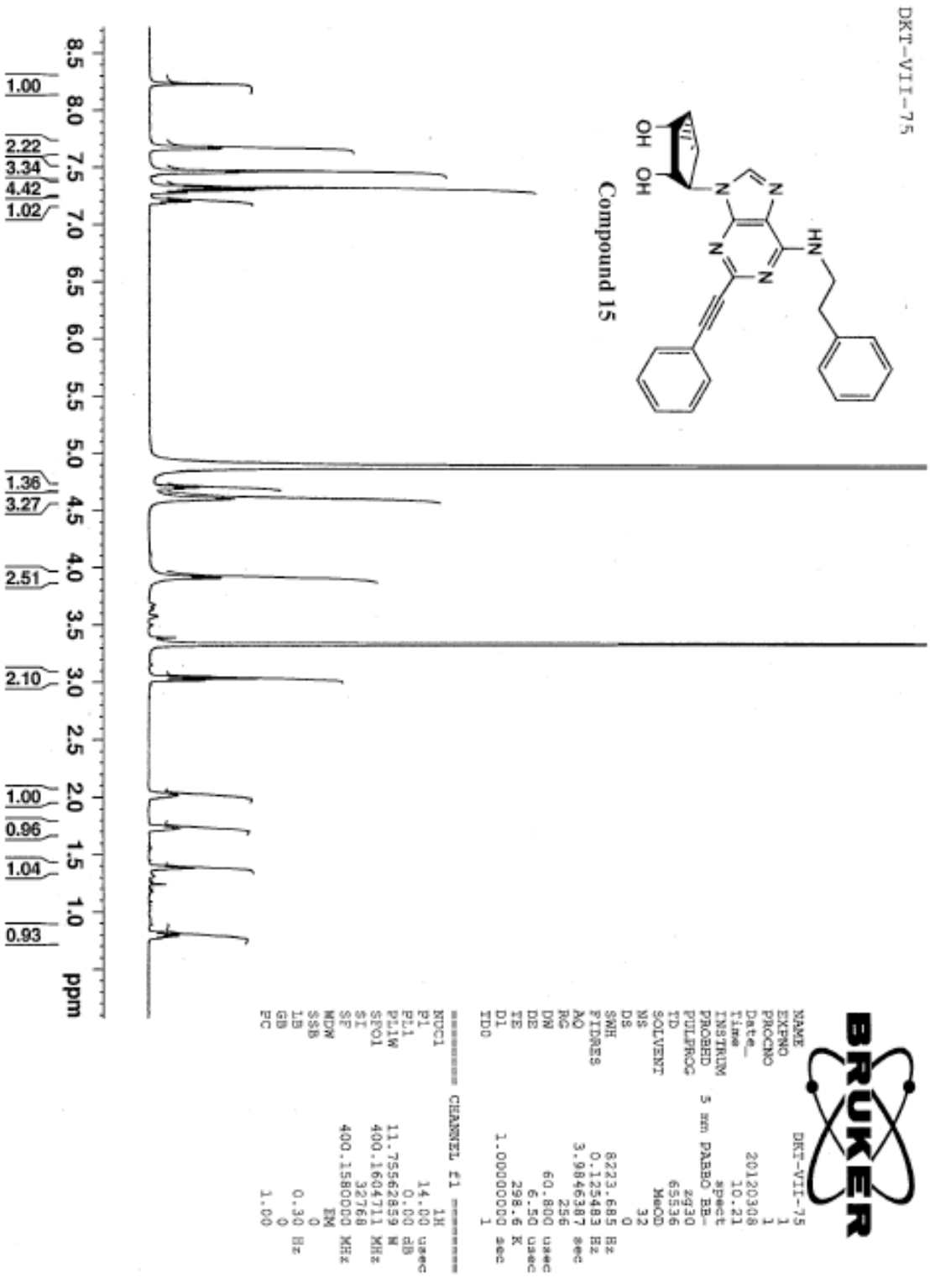


Compound 9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FIDRES 0.125483 Hz
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DE 6.50 usec
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SF 400.1580000 MHz
WDW EM
SSB 0
LB 0.30 Hz
GB 0
PC 1.00
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ppm
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1.02
1.36
3.27
2.51
2.10
1.00
0.96
1.04
0.93

Elemental Composition Report **Page 1**

Single Mass Analysis
Tolerance = 5.0 mDa / DBE: min = -1.5, max = 50.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 3

Monoisotopic Mass, Even Electron Ions
270 formula(e) evaluated with 2 results within limits (up to 50 closest results for each mass)
Elements Used:
C: 0-500 H: 0-1000 N: 0-6 O: 0-6

08-Mar-2012 09:52:09
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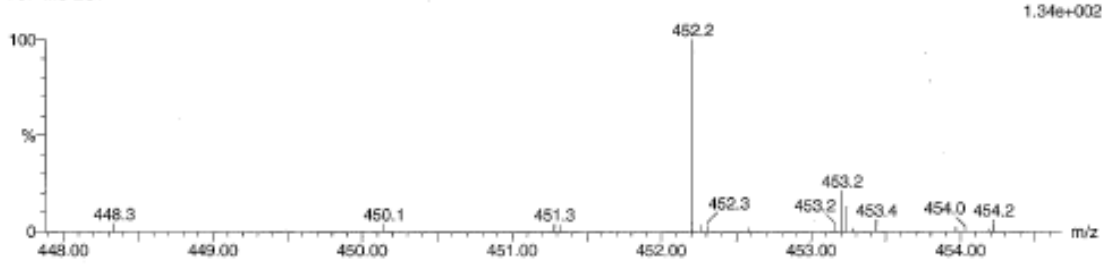
1.34e+002



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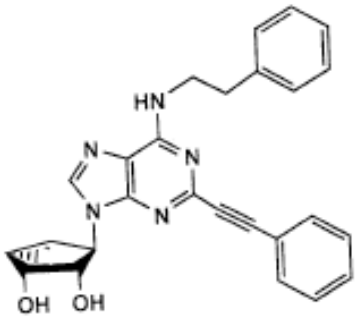

Compound 15

Pharmacological assays

Receptor binding and functional assays

[^{3}H]R-*N*^{6}-Phenylisopropyladenosine (**49**, [^{3}H]R-PIA, 63 Ci/mmol) and [^{125}I]*N*^{6}-(4-Amino-3-iodobenzyl)adenosine-5′-*N*-methyluronamide (**51**, [^{125}I]I-AB-MECA, 2200 Ci/mmol) were purchased from Perkin–Elmer Life and Analytical Science (Boston, MA). [^{3}H](2-[p-(2-Carboxyethyl)phenyl-ethylamino]-5′-*N*-ethylcarboxamido-adenosine) (**50**, [^{3}H]CGS21680, 39 Ci/mmol) was purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO). Other pharmacological reagents were purchased from Tocris-R&D Systems, Inc. (Minneapolis, MN). Test compounds were prepared as 5 mM stock solutions in DMSO and stored frozen.

Cell Culture and Membrane Preparation - CHO cells stably expressing the recombinant human (h) A_1 and hA_3ARs, and HEK-293 cells stably expressing the hA_{2A}AR were cultured in Dulbecco's modified Eagle medium (DMEM) and F12 (1:1) supplemented with 10% fetal bovine serum, 100 units/mL penicillin, 100 μg/mL streptomycin, and 2 μmol/mL glutamine. In addition, 800 μg/mL geneticin was added to the A_{2A} media, while 500 μg/mL hygromycin was added to the A_1 and A_3 media. After harvesting, cells were homogenized and suspended in PBS. Cells were then centrifuged at 240 *g* for 5 min, and the pellet was resuspended in 50 mM Tris-HCl buffer (pH 7.5) containing 10 mM $MgCl_2$. The suspension was homogenized and was then ultra-centrifuged at 14,330 *g* for 30 min at 4 °C. The resultant pellets were resuspended in Tris buffer, incubated with adenosine deaminase (3 units/mL) for 30 min at 37 °C. The suspension was homogenized with an electric homogenizer for 10 sec, pipetted into 1 mL vials and then stored at -80 °C until the binding experiments. The protein concentration was measured using the BCA Protein Assay Kit from Pierce Biotechnology, Inc. (Rockford, IL).[1]

Binding assays: Standard radioligand binding assays for hA_1, A_{2A}, and A_3ARs were used.[2-4] Into each tube in the binding assay was added 50 μL of increasing concentrations of the test ligand in Tris-HCl buffer (50 mM, pH 7.5) containing 10 mM $MgCl_2$, 50 μL of the appropriate agonist radioligand, and finally 100 μL of membrane suspension. For the A_1AR (22 μg of protein/tube) the radioligand used was [^{3}H]**49** (*R*-

PIA, final concentration of 3.5 nM). For the A_{2A}AR (20 μg/tube) the radioligand used was [^{3}H]**50** (CGS21680, 10 nM). For the A_3AR (21 μg/tube) the radioligand used was [^{125}I]**51** (I-AB-MECA, 0.2 nM). Nonspecific binding was determined using a final concentration of 10 μM NECA **52** diluted with the buffer. The mixtures were incubated at 25 °C for 60 min in a shaking water bath. Binding reactions were terminated by filtration through Brandel GF/B filters under a reduced pressure using a M-24 cell harvester (Brandel, Gaithersburg, MD). Filters were washed three times with 3 mL of 50 mM ice-cold Tris-HCl buffer (pH 7.5). Filters for A_1 and A_{2A}AR binding were placed in scintillation vials containing 5 mL of Hydrofluor scintillation buffer and counted using a Perkin Elmer Liquid Scintillation Analyzer (Tri-Carb 2810TR). Filters for A_3AR binding were counted using a Packard Cobra II γ-counter. The K_i values were determined using GraphPad Prism for all assays.

cAMP accumulation assay: Archival hA_3AR efficacy data for compounds **1** – **3**: Intracellular cAMP levels were measured with a competitive protein binding method.[5] CHO cells that expressed the recombinant hA_3AR were harvested by trypsinization. After centrifugation and resuspended in medium, cells were planted in 24-well plates in 1.0 mL medium. After 24 h, the medium was removed and cells were washed three times with 1 mL DMEM, containing 50 mM HEPES, pH 7.4. Cells were then treated with the agonist **52** (NECA) or test compound in the presence of rolipram (10 μM) and adenosine deaminase (ADA, 3 units/mL). After 45 min forskolin (10 μM) was added to the medium, and incubation was continued for an additional 15 min. The reaction was terminated by removing the supernatant, and cells were lysed upon the addition of 200 μL of 0.1 M ice-cold HCl. The cell lysate was resuspended and stored at -20°C. For determination of cAMP production, 100 μL of the HCl solution was used in the Sigma Direct cAMP Enzyme Immunoassay following the instructions provided with the kit. The results were interpreted using a Bio-Tek Instruments ELx808 Ultra Microplate reader at 405 nm.

hA_3AR efficacy data for new compounds **4** – **21**: CHO cells stably expressing the human A_3AR were seeded in 96-well plates and cultured overnight at 37 °C in 5% CO_2. The culture medium was replaced with stimulation buffer containing 0.1% bovine serum albumin (BSA), 3 units/mL ADA, and 10 μM rolipram, and incubated at 37 °C for 30 min. Agonists were then added and incubated for 30 min, followed by treatment with 10

μM forskolin for another 15 min, the medium was aspirated, and cells were lysed using lysis buffer (containing 0.3% Tween-20, 5 mM HEPES and 0.1% BSA). Samples were transferred to a 384-well plate. Donor bead-conjugated anti-cAMP antibody and biotinylated cAMP/acceptor bead-conjugated streptavidin were added according to instructions from cyclic AMP Alphascreen protocol. Samples were measured using the EnVision® Multilabel Reader (PerkinElmer). When used at a single concentration of 10 μM, the inhibition was expressed as a percent of the maximal effect of 10 μM 5′-*N*-ethylcarboxamidoadenosine (=100%).

Relative efficacy data at $hA_{2B}AR$ for selected compounds **3c**, **3e**, **5**, **6**, **9**, **10**, **13**, and **15** at a single concentration (10 μM) were evaluated. Stimulation of cyclic AMP production (expressed as % of full agonist 5′-*N*-ethylcarboxamidoadenosine, 10 μM) was determined using the cyclic AMP Alphascreen protocol, as above, with CHO cells stably expressing the $hA_{2B}AR$. Similar values (% relative efficacy at $hA_{2B}AR$) for other non-truncated nucleosides similar to **3** in this study, as reported by Tosh et al. (2012),[7] were: **15** (N^6-methyl, C2-(3-chlorophenyl)ethynyl), 21±4; **23** (N^6-methyl, C2-(4-acetylphenyl)ethynyl), 22±3; **28** (N^6-(3-chlorobenzyl), C2-(4-fluorophenyl)ethynyl), 26±1; **35** (N^6-(3-chlorobenzyl), C2-(pyren-1-yl)ethynyl), 24±3.

Data analysis

Binding and functional parameters were calculated using Prism 5.0 software (GraphPAD, San Diego, CA, USA). IC_{50} values obtained from binding inhibition curves were converted to K_i values using the Cheng-Prusoff equation.[6] Data were expressed as mean ± standard error.

1. Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **1976**, *72*, 248-254.
2. Klotz, K. N., Lohse, M. J., Schwabe, U., Cristalli, G., Vittori, S., and Grifantini, M. *Naunyn Schmiedebergs Arch. Pharmacol.* **1989**, *340*, 679-683.
3. Jarvis, M. F., Schutz, R., Hutchison, A. J., Do, E., Sills, M. A., Williams, M. *J.*

Pharmacol. Exp. Ther. **1989**, *251*, 888-893.

4. Olah, M. E., Gallo-Rodriguez, C., Jacobson, K. A., Stiles, G. L. *Mol. Pharmacol.* **1994**, *45*, 978-982.
5. Nordstedt, C., Fredholm, B. B. *Anal. Biochem.* **1990**, *189,* 231-234.
6. Cheng, Y. C., Prusoff, H. R. *Biochem. Pharmacol.* **1973**, *22*, 3099.
7. Tosh, D.K., Deflorian, F., Phan, K., Gao, Z.G., Wan, T.C., Gizewski, E., Auchampach, J.A., Jacobson, K.A. Structure-guided design of A_3 adenosine receptor-selective nucleosides: Combination of 2-arylethynyl and bicyclo[3.1.0]hexane substitutions. *J. Med. Chem.* **2012**, *55*, 4847-4860.

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Molecular modeling simulations were performed to study the putative interactions between the newly synthesized truncated (N)-methanocarba nucleosides (compounds **4**-**21**) and the residues of the hA_3AR binding site. Methodological details and procedures[1-7] are described below.

As previously reported also for the 5'-N-methyluronamido series,[8] these compounds were not able to fit inside the binding pocket of the hA_3AR model based on the agonist-bound $hA_{2A}AR$ X-ray structure (PDB code 3QAK)[2] due to the presence of bulky substituents at the C2 position (no reasonable docking poses). For this reason, we performed docking studies on the previously reported homology model of the hA_3AR based on a hybrid template where the extracellular part of TM2 was modeled following the structure of the opsin in the activated state (PDB code 3DQB).[5]

In this hybrid model, the extracellular terminal of TM2 was shifted outward by approximately 7 Å, as compared to the hA_3AR model based exclusively on the $A_{2A}AR$ crystal structure, with the creation of a larger pocket for the accessibility of C2 substituents of the present (N)-methanocarba nucleosides. This TM2 shift, observed in other agonist-bound or activated GPCRs, is supposed to be specific for adenosine ligands containing a rigid C2 extension, and it can occur in the hA_3AR thanks to the absence of structural constraints in that region. On the contrary, at the $A_{2A}AR$ the presence of two disulfide bridges between EL1 and EL2, in addition to the disulfide bond involving TM3 and EL2 and highly conserved among family A GPCRs, forces the extracellular terminal of TM2 toward the TM bundle. Therefore, the inability of the $A_{2A}AR$ to undergo this rearrangement might contribute to the A_3AR selectivity of these compounds.

Figure 1 shows the hypothetical binding mode of compound **10** inside the hA_3AR hybrid model binding pocket. Most of the key interactions previously observed for the 5'-N-methyluronamido series at this receptor were also noted for the docking pose of this compound.[8]

In particular, the 3′- and 2′-hydroxyl groups formed H-bonds with Ser271 (7.42) and His272 (7.43) side chains, respectively. The side chain of Asn250 (6.55) strongly interacted with compound **10** through two H-bonds involving the 6-amino group and the N^7 atom of the adenine ring. Moreover, the adenine ring was anchored inside the binding site by a π–π stacking interaction with Phe168 (EL2) and strong hydrophobic contacts with Leu246 (6.51) and Ile268 (7.39).

In the binding pose of compound **10**, the substituents at the N^6 and C2 positions were located in the extracellular portion of the hA_3AR binding pocket. In particular, the N^6-3-chlorobenzyl ring was accommodated in a hydrophobic pocket among TM5, TM6, and EL2 delimited by Val169 (EL2), Met174(5.35) and Ile253 (6.58), forming strong hydrophobic interactions with these residues.

On the other hand, the arylethynyl substituent at the C2 position of **10** was oriented toward TM2 and EL1 and was anchored by favorable hydrophobic interactions with residues of the upper part of the binding site, namely, Tyr15(1.35), Val72 (2.64), Tyr265 (7.36), Ile268 (7.39), and the carbon chain of Gln167 (EL2).

Compared to the docking pose of the 5'-N-methyluronamido series,[8] the only difference observed in the binding mode of this new series was the lack of interaction with Thr94 (3.36) due to the 4'-truncation of the new (N)-methanocarba nucleosides. In fact, while the 5'-N-methyluronamides were able to form a H-bond with the side chain hydroxyl group of Thr94 (3.36), the absence of a substituent at the 4'-position prevented

this new series of compounds from interacting with this key residue. This missing interaction was considered the reason for the low efficacy profile of these 4'-truncated nucleosides. In fact, even though no mutagenesis data are available for position 3.36 at the hA_3 subtype, previous mutagenesis studies have shown the importance of this threonine residue in agonist but not antagonist binding at the hA_1 and hA_{2A} subtypes.[9,10] Therefore, interaction of ligands with Thr 3.36, conserved among all ARs, might be crucial to lock the ribose/methanocarba moiety in an optimal conformation to strongly interact with residues at positions 7.42 and 7.43 in order to pull TM7 towards TM3 to efficiently activate the receptor.

Overall, these molecular modeling results showed that the binding of the newly synthesized (N)-methanocarba nucleosides is allowed by the plasticity of the receptor structure and by its ligand-specific reorganization that creates a very large hydrophobic pocket, dependent on an outward displacement of TM2, able to accommodate bulky and rigid groups at the C2 position. Moreover, the lack of a key H-bond with Thr94 (3.36) and the consequent poor stabilization of the methanocarba ring seemed to be the reason for the low efficacy profile of these derivatives. However, these ligands were able to form strong interactions with many residue already hypothesized to be involved in the binding of other hA_3AR antagonists,[11] such as Asn250 (6.55), Phe168 (EL2), Leu246 (6.51) and Val169 (EL2). These findings are consistent with the pharmacological results showing for these (N)-methanocarba nucleosides good affinity but low efficacy at the hA_3AR.

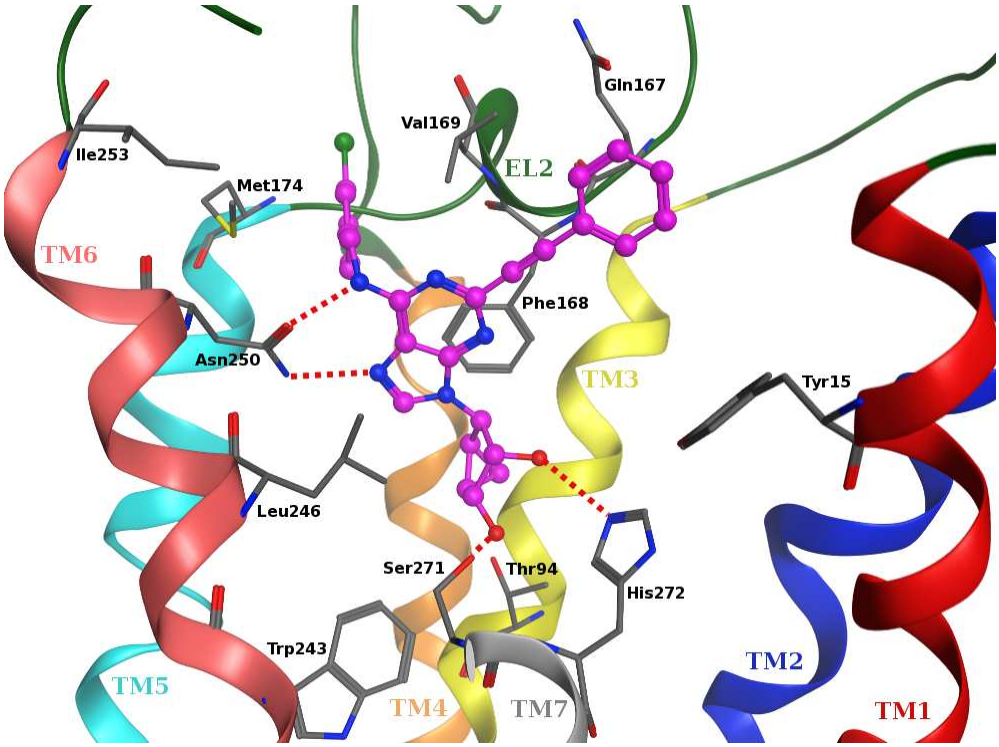

Figure 1: Docking pose of compound **10** (in magenta) inside the binding site of the hA_3AR hybrid model. Side chains of some amino acids important for ligand recognition and H-bonding interactions are highlighted. Hydrogen atoms are not displayed. Part of TM7 is omitted.

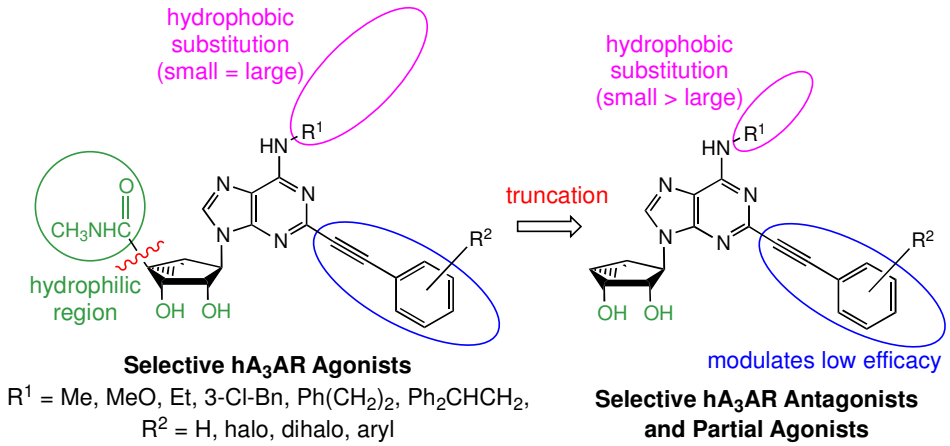

Figure 2: General requirements of high affinity binding to the hA_3AR, comparing 5'-N-methyluronamido series (on the left) and 4'-truncated series (on the right). In the truncated series, smaller N^6 substitutuents, such as methyl and ethyl, provided higher A_3AR affinity than N^6-arylalkyl substitutuents.

Experimental section

Molecular modeling

hA_3AR homology models:

Previously reported molecular models of the hA_3AR, built using the alignment and the homology modeling tools implemented in the MOE suite,[1] were used in this study. In particular, two different models were taken into account. A first model was built using as template the crystal structure of the $A_{2A}AR$ co-crystallized with the agonist UK-342097 (PDB ID: 3QAK)[2], as described by Tosh et al.[3]

The second hA_3AR model was based on a hybrid template structure as previously published.[4] To build this model, the agonist-bound $A_{2A}AR$ crystal structure was used as template for the entire A_3AR structure except for the extracellular terminus of TM2 (residues from Val63 to Ser73 of A_3AR) and EL1 (residues from Leu74 to Tyr81 of A_3AR). The X-ray structure of the opsin in its active conformation (PDB ID: 3DQB)[5] was used as template to build the extracellular terminus of TM2. During the homology modeling of this hybrid model of A_3AR, no structural template was used for the modeling of EL1.

Molecular docking of truncated (N)-methanocarba derivatives at the hA_3AR models:

Compounds structures were built using the builder tool implemented in the MOE suite[1] and subjected to energy minimization using the MMFF94x force field, until a RMS gradient of 0.05.

Molecular docking of the ligands at the hA_3AR models was performed by means of the Glide[6] package part of the Schrödinger suite.[7]

The docking site was defined with key residues in the binding pocket of the A_3AR model, namely Phe168, Asn250, Trp243 and His272, and a 26 Å x26 Å x26 Å box was centered on those residues. Docking of the ligands was performed in the rigid binding site

with Glide 5.0 using the XP (extra precision) procedure. The top scoring docking conformations were subjected to the receptor sampling by means of the Refinement module in Prime 2.0. The Prime side-chain sampling was performed on all the residues within a 6Å of the ligand. The refined model for each ligand was chosen as final binding conformation.

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