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Synthesis and Evaluation of Nicotinic Acid Derived Tetrazines for Bioorthogonal Labeling

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Abstract: A set of nicotinic acid derived tetrazines were synthesized and evaluated for activity in inverse electron demand Diels-Alder (IEDDA) reactions with various dienophiles. It was found that the performance of these tetrazines is governed by two factors. Theoretical and experimental investigations showed that steric effects may override the energetically predicted order of reactivity. Making a compromise between reactivity and stability a selected tetrazine was incorporated into a deoxynucleotide to afford a bioorthogonalized bulding block enabling IEDDA based tagging schemes of nucleic acids.

Key words: tetrazine, inverse electron demand Diels-Alder, bioorthogonal, nucleic acid, labeling

Chemical manipulation, e.g. fluorescent tagging of biomolecules by means of inverse electron demand Diels-Alder (IEDDA) reaction between tetrazines and strained ring systems present the fastest bioorthogonal modulation procedure today.[1] Accordingly, selective and efficient labelling of DNA[2,3] and RNA[4] was achieved by activated olefins as building blocks, introduced either as phosphoramidites or nucleoside triphosphates. Most recently, it was shown that this type of DNA modification chemistry works also in living cells.[3] Orthogonality of such IEDDA reactions with other bioorthogonal transformations for example, with strain promoted azide-alkyne cycloaddition (SPAAC) was also examined.[5] In fact, one-pot dual labeling by IEDDA and copper-catalyzed cycloaddition was already realized for modification of DNA.[6] Orthogonal modification of biomatter by the same bioorthogonal transformation is also possible. Lemke and co-workers demonstrated that tetrazines with distinct reactivities towards dienophiles enable pulse-chase labeling of insulin receptors expressing non-canonical amino acids that harbor different dienophiles.[5] In this latter report the distinct reactivity of a non-symmetrically and a mono-substituted tetrazine implies the governing effects of the steric demand of the substituents in the proximity of the reaction center.

For our purposes that involve the development of bioorthogonalized oligonucleotides we have sought for new tetrazine derivatives with reasonable reactivities and stability suitable for longer *in vivo* exposure. Reactivity and stability are two seemingly contradictory properties, and in most cases a compromise is necessary when selecting biorthogonally applicable reagents. Former examples show that the reactivity of tetrazines can be predicted with relatively high fidelity as it is mainly governed by the energy of its frontier molecular orbital (LUMO).[7] As a rule of thumb, electron withdrawing substituents lower the energy of the LUMO of the tetrazine scaffold therefore increase reactivity.[7]

The presence of pyridine units appending from the tetrazine core is often beneficial for the reactivity of tetrazines towards dienophiles.[1,7] For its synthetic accessibility, further functionalization and its rate enhancing features, we have chosen nicotinic acid based nitriles to build up activated tetrazine scaffolds. We were also curious of inspecting the effect of steric demand of the substituents on the reaction rate. Therefore we have designed three nicotinic amide modified tetrazines as model compounds in order to evaluate their reactivity towards selected dienophiles (Figure 1).

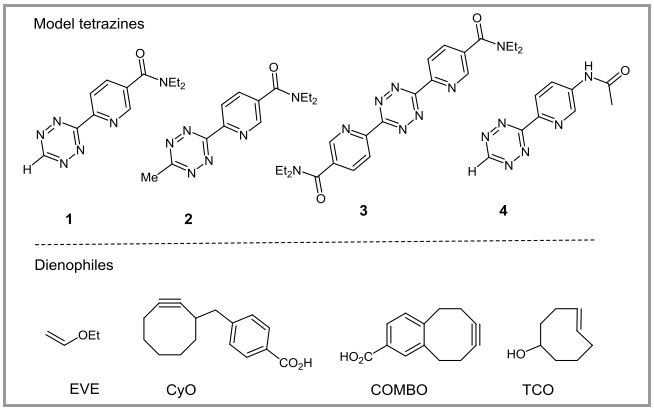
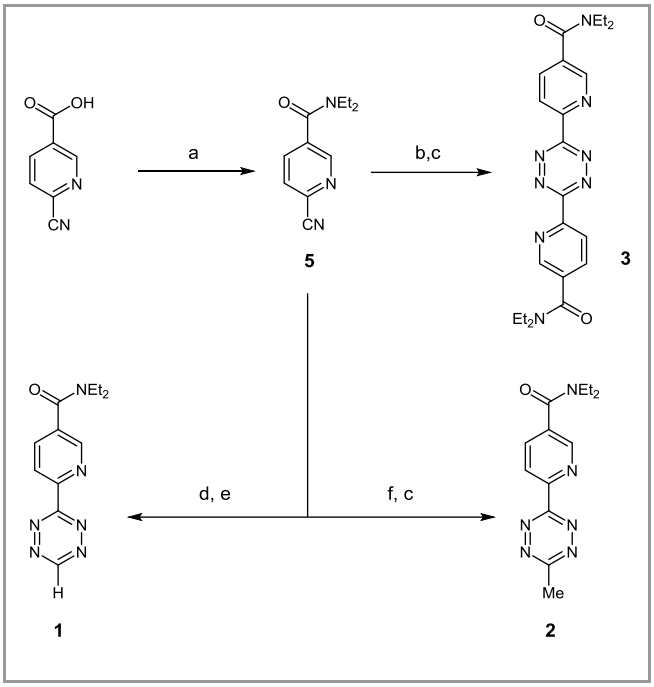


Figure 1 Structure of model tetrazines with different steric demand (top) and dienophiles used in this study (bottom).

The synthesis of monosubstituted tetrazine (**1**) started with the reaction of 6-cyano-*N*,*N*-diethylnicotinamide (**5**) with aqueous hydrazine in the presence of formamidine acetate. The resulting dihydrotetrazine was oxidized directly with isopentyl-nitrite to furnish **1** in 42% yield (2 steps). Non-symmetrically substituted tetrazine (**2**) was made in a similar manner from **5** and acetonitrile in the presence of hydrazine-hydrate. The

primary product dihydrotetrazine was treated with *in situ* generated nitrous gases to provide **2** in an overall 10% yield. Symmetrically decorated tetrazine (**3**) was obtained by treating 6-cyano-*N*,*N*-diethylnicotinamide with hydrazine-hydrate. Subsequent oxidation with nitrous gases yielded **3** in an overall 30% yield (Scheme 1).



Scheme 1 Synthesis of model tetrazines. a) $HNEt_2$, HOBt, HBTU, DIPEA, THF, r.t. 16h, 87%; b) $N_2H_4.H_2O$, THF, Δ, 16h; c) [NO_x], CH_2Cl_2, r.t. 15 min, 30% (**3**), 10% (**2**) (2 steps); d) formamidine acetate, $N_2H_4.H_2O$, EtOH, Δ, 16h; e) isopentyl nitrite, CH_2Cl_2, r.t. 3h, 42%; f) $N_2H_4.H_2O$, MeCN, Δ, 16h .

With these model compounds in hand we were curious how these tetrazines react with simple, sterically less demanding - and less reactive - dienophiles like ethyl-vinyl ether (**EVE**). For comparison, we have used dipyridyl tetrazine (**DPT**)[8] as standard. In order to mimic intracellular conditions, the reactions were accomplished under pseudo-first order conditions. Evaluation of the results showed that symmetrically substituted **3** and monosubstituted **1** react with EVE at comparable speeds (k_2 = 8 and 24 $\times 10^{-3}$ $M^{-1}s^{-1}$, respectively), while compound **2** and **DPT** gave k_2 values an order of magnitude lower. It should be noted that this order of rate constants is not in line with the reactivity order predicted by the LUMO energies (Table 1) and confirms our belief that steric demand of the substituents can also contribute to the overall reactivity.

To track down the effects of steric demand we have conducted another series of experiments where tetrazines **1** and **3** were reacted with a more reactive but bulkier dienophile (**CyO**)[9]. In this case the difference in rate constants was even more profound. Monosubstituted **1** reacted ca. 30 times faster than energetically favored symmetrical tetrazine **3**.

Table 1 Second order rate constants of the reaction between tetrazines and various dienophiles. The LUMO energies were calculated at the Hartree–Fock level of theory with the 6-311++G** basis set at B3LYP/6-311++G** geometries

tetrazine	$t_{1/2}$ (h)	LUMO (eV)	k_2 / $M^{-1}s^{-1}$			
			EVE[a]	CyO[a]	COMBO[b]	TCO[b]
DPT	3[10]	0.28	0.0012	---	---	---
1	4	0.71	0.024	6.25	---	---
2	88	0.83	0.00145	---	210	4000
3	4	0.46	0.008	0.23	---	---
4	6	0.69	0.010	2.89	---	---

a) measured in DMSO/water (9/1 v/v) at 25 °C; b) measured in 1% DMSO in PBS buffer (pH 7.2) at 36 °C.

To verify our findings we have also run theoretical calculations involving steric demand of reactants in transition states for the selected reactions. The geometries of the tetrazines and dienophiles as well as the corresponding transition states were optimized at the density functional (DFT) level using the B3LYP (Becke 3-parameter Lee-Yang-Parr exchange-correlation) functional[11] and the 6-311++G** basis set. To save computer time the ethyl groups of the tetrazine derivatives were replaced by methyl groups. For each molecule considered, several stable conformers exist, which slightly differ in energy. The geometry of all conformers was optimized, and the lowest-energy one was considered in the subsequent transition state searches.

Table 2 Gibbs energies of activation ($\Delta G^{\ddagger}$, kcal/mol) for the reactions of tetrazines **1** and **3** with ethyl-vinyl ether and **CyO** evaluated at the B3LYP level with the 6-311++G** basis set

Tetrazine	Ethyl-vinyl ether	CyO
1	31.5	24.4
3	33.4	29.4

Furthermore, except for the reaction of **3** with **EVE** several transition state structures can be located. We determined the activation barriers for all the possible structures, but only the transition states with the lowest barriers are considered here. At the optimized geometries harmonic vibrational frequencies were evaluated and Gibbs energies of activation were computed in the rigid rotor-harmonic oscillator approximation. All calculations were carried out with the Gaussian 09 suite of quantum chemistry programs[12].

The results are compiled in Table 2, while in Figure 2 the transition-state geometries are visualized for the reactions of **1** and **3** with **CyO**. To our delight, data are in good correlations with the experimental results. The free energies of activation are higher for compound **3** with both dienophiles. In addition, the difference between the activation barriers for the reactions of **1** and **3** is somewhat bigger for **CyO**. As can be seen in Figure 2 the methyl group of **CyO** is rather close to the hydrogen atom at the pyridine substitutent. Thus the

steric repulsion is considerably stronger between the derivative substituted with the bulky pyridine group and **CyO**, which explains the observed behavior of the compounds.

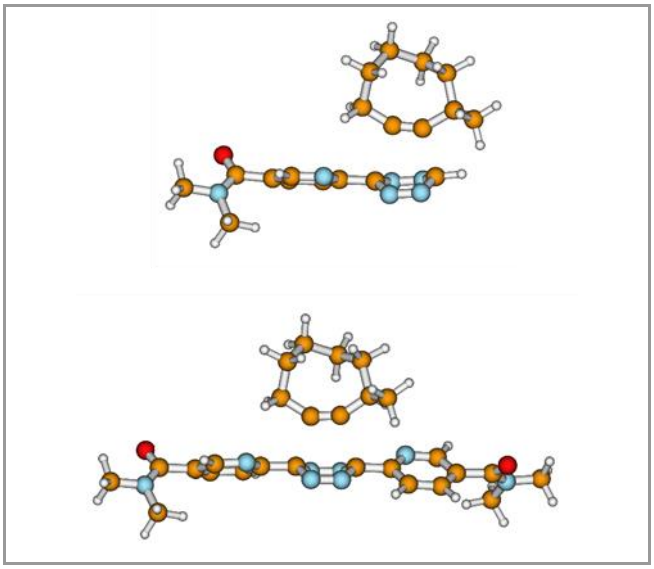


Figure 2 Optimized transition-state structures for the **1** + **CyO** (top) and the **3** + **CyO** (bottom) reactions.

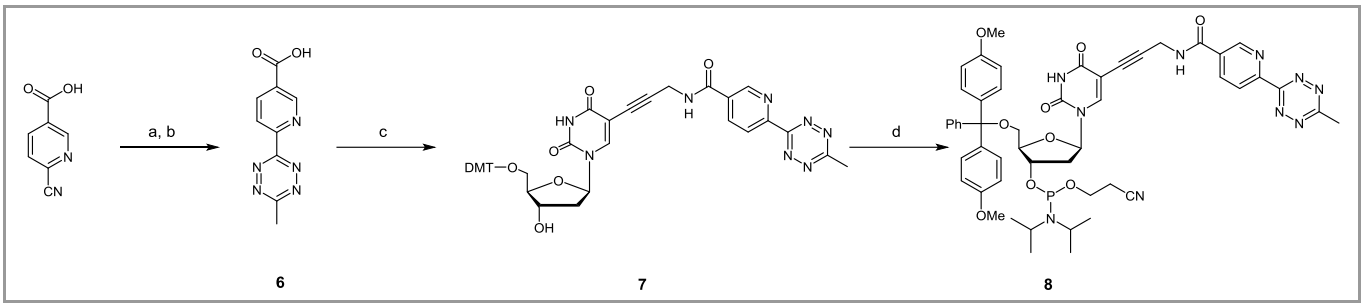
Next we have examined the stability of the tetrazines by determining their half-lives in aqueous solutions (pH 7.2 PBS). As expected, the stability was found to be inversely proportional to reactivity. Whilst **1** reacted the fastest with all dienophiles it decomposed in aqueous solutions relatively rapidly. Tetrazine **2** on the other hand was found to be quite stable under similar conditions.

We have attempted to preserve reactivity in a more stable tetrazine scaffold. Thus we have synthesized tetrazine derivative **4** (see supporting information). We have anticipated that compound **4** would be more stable yet with comparable in reactivity to **1**. The synthesis of **4** was accomplished similarly to that of **1**. Acetylation of 5-aminopicolinonitrile with acetyl chloride and subsequent reaction with formamidine acetate in the presence of hydrazine hydrate followed by oxidation with isopentyl nitrite provided model tetrazine **4** in medium yields (see supporting information). While the second order rate constant of **4** was determined to be as expected its stability turned out to be only slightly better than **1**.

Our continuing efforts on DNA modification studies[13] require bioorthogonal handles stable enough to survive solid phase oligonucleotide synthesis (SPOS) or stand *in vivo* conditions. Thus, we have sacrificed reactivity for stability and chose tetrazine **2** for further studies. To judge the reactivity of **2** we have conducted kinetic studies in the presence of highly strained and reactive dienophiles. To our delight, monobenzocyclooctyne (**COMBO**)[14] and *trans*-cyclooctene (**TCO**)[15] reacted quite rapidly with **2** (Table 1) justifying its use in reasonably fast bioorthogonal labeling schemes. The reactivity of **2** with **COMBO** is in good accordance with similar studies with a structurally related derivative of **2** with another, less reactive strained cyclooctyne **BCN**[16] ($k_{2(COMBO)}$ = 210 $M^{-1}s^{-1}$ vs. $k_{2(BCN)}$ = 80 $M^{-1}s^{-1}$).[17]

Next, we aimed at incorporating non-symmetrically substituted tetrazine **2** into a deoxyuridine-phosphoramidite building block. It bears the tetrazine unit linked by an aminopropynyl group at the 5-position. This has two advantages: Firstly, it potentially allows to use this nucleoside conjugate for primer extension and PCR since commonly applied DNA polymerases like KlenTaq polymerase typically tolerate such modifications.[18] Secondly, the structural influence by this modification should be rather small. For this, we treated cyanonicotinic acid with hydrazine hydrate in the presence of triethyl orthoacetate (Scheme 2). Subsequent oxidation with $NaNO_2$ / HCl furnished tetrazine **6**. Parallel to this, commercially available 5-iodo-2'-deoxyuridine was subjected to a Sonogashira-type coupling reaction with trifluoroacetate protected propargylamine.[13,19] After removal of the protecting group in aqueous ammonia the resulting amino functionalized 2'-deoxyuridine was reacted with **6** in the presence of HBTU / HOBt and Hünig's base. The tetrazine modified 2'-deoxyuridine **7** was converted to its phosphoramidite using 2-cyanoethyl-*N,N*-di*iso*propylchlorophosphoramidite to furnish phosphoramidite **8** (Scheme 2).

Tetrazine modified 2'-deoxyuridine phosphoramidite (**8**) was incorporated into a model oligonucleic acid **DNA1** using standard SPOS methods. The synthetic protocol was modified such that the removal of the last DMT-protecting group from the complete sequence was followed by an automated on-bead labeling step applying a reactive cyclooctyne derivatized fluorescein dye (**COMBO-Flu**, Figure 3)[14] for 60 s. Mass spectrometry of the labeled oligonucleotide verified the existence of the right product. Spectrophotometric analysis of the labeled DNA showed the characteristic absorption and emission bands of fluorescein at around 500 and 520 nm, respectively. Upon hybridization of the labeled strand with its complementary strand, **DNA1c**, an increase of extinction, especially of the band at 500 nm, could be observed (see supporting information). This increase can be explained by unshielding the dye from the DNA bases upon annealing the randomly folded single stranded DNA.[13] Formation of a structured double-stranded DNA presumably forced the fluorescein moiety outside the DNA-helix and face towards the more polar aqueous environment. This is supported by the melting temperatures that show only a slight decrease relative to that of the unmodified DNA (56.8 °C vs. 59.5 °C).



Scheme 2 Synthesis of bioorthogonalized 2'deoxyuridine-phosphoramidite **8**. a) triethyl orthoacetate, $N_2H_4 \times H_2O$, Δ, 2h; b) NO_x, CH_2Cl_2, rt., 3h, 25% (for 2 steps). c) DMT-dU-propargylamine, HBTU, HOBt, DIPEA, DMF, 16h, 65% d) 2-cyanoethyl-N,N-diisopropylchloro phosphoramidite, DIPEA, CH_2Cl_2, 89%.

An on-bead labeling typically does not allow to determine yields of coupling efficiencies. However, the incorporation efficiency of the tetrazine modified building block was comparable to that of the commercial building blocks for A, G, T and C.

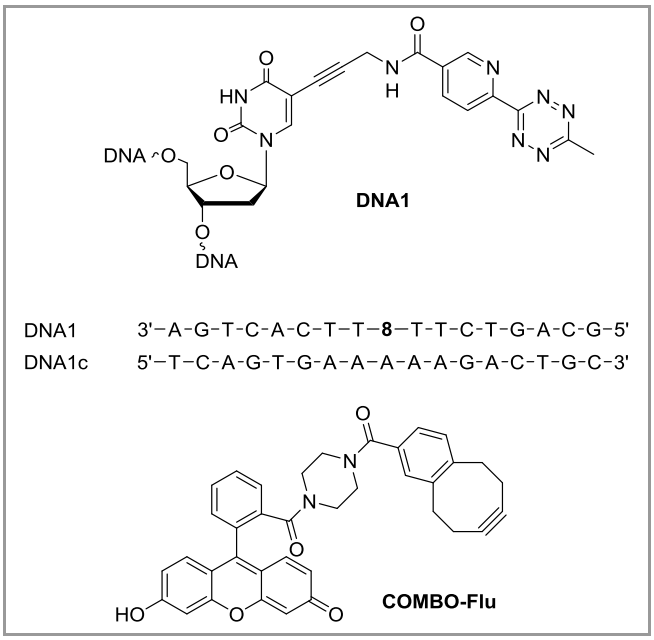


Figure 3 Sequence of DNA1 with the tetrazine building block **8** that reacts rapidly with the cycloocytyne-modified fluoresceine (COMBO-Flu).

This study revealed that steric demand of substituents play prominent role in the Diels-Alder reactivity of electron deficient tetrazines. We have investigated the reaction rates of model tetrazines in the presence of various dienophiles. The experimental findings were supported by theoretical investigations as well. Besides reactivity, the stability of these new tetrazines was also studied and it was found that only the least reactive, non-symmetrically decorated tetrazine can stand physiological conditions for longer times. For our purposes, we therefore used this scaffold in the design of a bioorthogonalized nucleic acid building block, **8**. Incorporation of this modified building block into a DNA sequence using standard solid phase nucleic acid synthesis, a fully automated protocol together with subsequent on-bead labeling experiment revealed that this tetrazine is suitable for the implementation of a reactive bioorthogonal handle into oligonucleotides.

Though in this study we have sought for stable tetrazines for incorporation into biomolecular building blocks, the more reactive tetrazines developed in this study can be used e.g. for labeling purposes where exposure times range only between 0.1 – 2 h. Related labeling studies with reactive tetrazines as well as attempts to synthesize a nucleotide-triphosphate derivative of **8** in order to test the suitability of this unnatural building block in PCR applications are in progress and results will be reported in due course.

General:

Unless otherwise indicated, all starting materials were obtained from commercial suppliers (Sigma-Aldrich, Fluka, Merck, Alfa Aesar, Reanal, Molar Chemicals) and used without further purification. Dichloromethane stabilized with ethanol was used, unless otherwise mentioned. CyO, COMBO and TCO were synthesized according to literature procedures.[9,14,15] Analytical thin-layer chromatography (TLC) was performed on silica gel 60 F254 precoated aluminium TLC plates from Merck. Column chromatography was carried out with flash silica gel (0.040–0.063 mm) from Molar Chemicals. Visualization of TLC samples was performed with a 254/365 nm UV. NMR spectra were recorded on a Bruker Avance 250 MHz, Bruker Avance III 400 MHz or a Varian Inova 600 MHz spectrometer. Chemical shifts (δ) are given in parts per million (ppm) using solvent signals as the reference. Coupling constants (J) are reported in Hertz (Hz). Splitting patterns are designated as s (singlet), d (doublet), t (triplet), qr (quadruplet), qv (quintuplet), m (multiplet), dd (doublet of a doublet), td (triplet doublet), dt (doublet triplet), br s (broad singlet). The exact masses were determined with an Agilent 6230 time-of-flight mass spectrometer. All melting points were measured on a Bibby Scientific SMP10 Melting Point Apparatus and are uncorrected. IR spectra were obtained on a

Bruker Alpha-P spectrometer on a single-reflection diamond ATR unit. The fluorescence measurements were carried out on a Fluoromax 4 (Horiba) spectrofluorimeter, the UV-VIS measurements on a Cary 100 (Varian) spectrophotometer.

6-cyano-N,N-diethylnicotinamide (5)[20]

To a solution of 6-cyanonicotinic acid (250 mg, 1.6 mmol) in THF (15 ml) were added HBTU (599 mg, 1.5 mmol), HOBt (257 mg, 1.6 mmol), DIPEA (604 µl, 3.5 mmol) and diethylamine (210 µl, 2.0 mmol). The resulting suspension was stirred at room temperature for 12 hours. After then, the volatiles were evaporated in vacuo. Purification of the product by column chromatography on silica gel with hexanes/EtOAc 3:1 v/v afforded **5** as a pale yellow oil in 87% yield.

^{1}HNMR ($CDCl_3$): δ= 8.66 (1H, d, J=1.2), 7.8 (1H, dd, J=7.9, 2.1), 7.7 (1H, dd, J=7.9, 0.7), 3.57-3.09 (4H, m), 1.20 (6H, m).

^{13}CNMR ($CDCl_3$): δ= 171.8, 147.2, 138.2, 131.8, 131.3, 123.2, 116.2, 41.9, 13.15.

N,N-diethyl-6-(1,2,4,5-tetrazin-3-yl)nicotinamide (**1**)

Formamidine acetate (2.95 g, 28.32 mmol, 12 eq.) was dissolved in hydrazine monohydrate (aqueous 64%, 10 mL) and heated to 60 °C. A solution of **5** (0.48 g, 2.36 mmol, 1 eq.) in anhydrous ethanol (20 mL) was added dropwise (15 min) to it and the reaction mixture was stirred overnight at 80 °C. After evaporation of the solvent the remaining sticky residue was dissolved in EtOAc (200 mL), washed with 10 m/m% citric-acid solution (2×50 mL), cc. $NaHCO_3$ solution (2×40 mL) and brine (30 mL) and dried over $MgSO_4$. The solution was filtered and the solvent was removed in vacuo to furnish crude dihydrotetrazine, which was dissolved in CH_2Cl_2 (150 mL). Isopentyl nitrite (950 µL, 7.08 mmol, 3 eq.) was added to the solution and the reaction mixture was stirred at room temperature for 3 h while the yellow solution turned deep red and TLC indicated the full consumption of the dihydrotetrazine. After evaporation of the solvent the crude product was purified by column chromatography (SiO_2, CH_2Cl_2/MeOH = 50/1 → 30/1) to yield 256 mg **1** (42%) as a purple-red solid.

T.l.c.: R_f = 0.4 (CH_2Cl_2/MeOH = 94/6 v/v).

M.p. = 222-225 °C (decomp.).

^{1}H NMR (250 MHz, $CDCl_3$) δ = 10.35 (s, 1H), 8.91 (d, J = 1.8 Hz, 1H), 8.70 (d, J = 8.1 Hz, 1H), 7.99 (dd, J = 8.1, 1.8 Hz, 1H), 3.56 (q, J = 6.5 Hz, 2H), 3.27 (q, J = 6.5 Hz, 2H), 1.25 (t, J = 6.5 Hz, 3H), 1.14 (t, J = 6.5 Hz, 3H).

^{13}C NMR (62.5 MHz, $CDCl_3$) δ = 167.4, 165.2, 158.3, 150.1 148.2, 135.8, 135.6, 123.9, 43.4, 39.6, 14.2, 12.7.

IR (neat): υ_{max} = 3074, 2936, 1618, 1429, 1344, 1096, 866.

HRMS (ESI) $[M+H]^+$ calcd. for $C_{12}H_{15}N_6O^+$: 259.1302, found: unable to acquire, decomposes.

N,N-diethyl-6-(6-methyl-1,2,4,5-tetrazine-3-yl)-nicotinamide (**2**)

5 (420 mg, 2.0 mmol) was dissolved in MeCN (20 ml). Hydrazine monohydrate (aqueous 64%, 511 µl, 10.25 mmol) was added to it dropwise and the solution was stirred at 90 °C for 12 hours. The volatiles were evaporated and the residue was dissolved in CH_2Cl_2. The solution was washed twice with citric acid solution (20 mL, 5% aqueous solution) and saturated Na_2CO_3 (20 mL). The organic phase was dried over $MgSO_4$, and filtered. Nitrous gases were bubbled through the solution for 10 minutes during which time the color of the solution turned deep pink. The solvent was evaporated and the crude product was purified by column chromatography on silica gel (CH_2Cl_2/MeOH 30:1 v/v). Further purification by recrystallization from ethanol yielded **2** as bright magenta crystals (9%).

Melting point: 177-179 °C (decomp.).

T.l.c.: R_f = 0.3 (CH_2Cl_2/MeOH 30:1 v/v).

^{1}HNMR (400 MHz, CD_3OD) δ = 8.85 (s, 1H), 8.75 (d, J = 7.8 Hz, 1H), 8.16 (d, J = 7.8 Hz, 1H), 3.62 (q, J = 6.4 Hz, 2H), 3.38 (q, J = 6.4 Hz, 2H), 3.13 (s, 3H), 1.30 (t, J = 6.4 Hz, 3H), 1.20 (t, J = 6.4 Hz, 3H)

^{13}CNMR (100 MHz, CD_3OD) δ = 169.9, 169.8, 164.1, 152.1, 148.8, 137.3, 136.7, 124.9, 45.1, 41.2, 21.3, 14.4, 13.1.

170.3, 170.1, 164.6, 149.3, 137.8, 137.2, 125.3, 41.8, 22.0, 13.6 ppm.

IR (neat): υ_{max} = 2979, 2967, 1617, 1403, 1363, 1285, 867 cm^{-1}.

^{13}CDEPT NMR (100 MHz, $CDCl_3$): δ = 169.9(-), 169.8(-), 164.1(-), 152.1(-), 148.8(+), 137.3(+), 136.7(-), 124.9(+), 45.1(-), 41.2(-), 21.3(+), 14.4(+), 13.1(+).

HRMS (ESI) $[M+Na]^+$ calcd. for $C_{13}H_{16}N_6ONa^+$: 295.1283, found: 295.1295.

6,6'-(1,2,4,5-tetrazine)-bis(N,N-diethylnicotinamide) (**3**)

5 (297 mg, 1.4 mmol) was dissolved in 10 ml THF. Aqueous hydrazine monohydrate (64%, 141 µl, 2.93 mmol) was added dropwise and the solution was stirred at 80 °C overnight. The THF was evaporated and the resulting crude dihydrotetrazine was redissolved in 150 ml of CH_2Cl_2 and oxidized directly with nitrous gases. The nitrous gases were bubbled through the stirred solution for 10 minutes. After evaporation of CH_2Cl_2 the crude product was purified by column chromatography on silica gel (CH_2Cl_2/MeOH 94:6 v/v). Further purification is possible by recrystallization from EtOAc to yield **3** as magenta crystals (30%).

T.l.c.: R_f = 0.7 (CH_2Cl_2/MeOH 94:6 v/v).

M.p.: 180-183 ^{0}C (decomp.).

^{1}HNMR (400 MHz, $CDCl_3$): δ = 8.98 (d, J = 1.4 Hz, 2H), 8.81 (d, J = 8.2 Hz, 2H), 8.04 (dd, J = 8.2, 1.4 Hz, 2H), 3.61 (q, J = 6.3 Hz, 4H), 3.33 (q, J = 6.3 Hz, 4H), 1.40-1.10 (m, 12H).

^{13}CNMR (100 MHz, $CDCl_3$): δ = 167.7, 163.6, 150.4, 148.6, 136.0, 135.8, 124.5, 43.6, 39.9, 14.5, 13.0.

IR (neat): υ_{max} = 1629, 1427, 1362, 1290, 1124, 857, 603 cm^{-1}.

^{13}CDEPT NMR (100 MHz, $CDCl_3$): δ = 167.7(-), 163.6(-), 150.4(-), 148.6(+), 136.0(+), 135.8(-), 124.5(+), 43.6(-), 39.9(-), 14.5(+), 13.0(+).

HRMS (ESI) $[M+Na]^+$ calcd. for $C_{22}H_{26}N_8O_2Na^+$: 457.2076, found: 457.2092.

6-(6-methyl-1,2,4,5-tetrazin-3-yl)nicotinic acid (**6**)

6-cyanonicotinic acid (593 mg, 4.00 mmol, 1 eq.), sulfur (90 mg, 2.80 mmol, 0.7 eq.) and triethyl orthoacetate (18.3 mL, 100 mmol, 25 eq.) was placed in a round bottomed flask and flushed with N_2. Hydrazine monohydrate (15 mL, 300 mmol, 75 eq.) was added and the reaction mixture was stirred vigorously at 100 °C for 2 h. After cooling to room temperature the solution was diluted with EtOAc (200 mL) and washed with 10 m/m% citric-acid solution (2×75 mL), cc. $NaHCO_3$ solution (2×50 mL), brine (30 mL) and dried over $MgSO_4$. The solution was filtered and the solvent was evaporated. Crude dihydrotetrazine was suspended in CH_2Cl_2 (150 mL) and oxidized with *in situ* generated nitrous gases (NO_x) till t.l.c. indicated the consumption of all dihydrotetrazine and the solution turned deep red. After evaporation of the solvent the crude product was purified by column chromatography (SiO_2, CH_2Cl_2/MeOH = 9/1 v/v) to yield 218 mg **6** (25%) as a purple-red solid.

T.l.c.: R_f = 0.2 (CH_2Cl_2/MeOH = 10/1 v/v).

M.p. = 158-161 °C (decomp.).

^{1}HNMR (250 MHz, DMSO-d_6) δ = 9.33 (dd, J = 2.1, 0.7 Hz, 1H), 8.64 (dd, J = 8.2, 0.7 Hz, 1H), 8.56 (dd, J = 8.2, 2.1 Hz, 1H), 3.06 (s, 3H).

^{13}CNMR (150 MHz, DMSO-d_6) δ = 167.7, 165.9, 162.7, 153.2, 150.8, 138.5, 128.6, 123.7, 21.0.

MS (ESI): [M-H]- calcd. for $C_9H_6N_5O_2^-$: 216.1, found: 216.0.

IR(neat): υ_{max} = 3051, 2850, 2552, 1680, 1402, 1247, 1020.

HRMS (ESI) $[M+Na]^+$ calcd. for $C_9H_7N_5O_2Na^+$: 240,0492, found: 240.0484.

N-(3-(1-((2R,4S,5R)-5-((bis(4-methoxyphenyl)(phenyl)methoxy)methyl)-4-hydroxytetrahydrofuran-2-yl)-2,4-dioxo-1,2,3,4-tetrahydropyrimidin-5-yl)prop-2-yn-1-yl)-6-(6-methyl-1,2,4,5-tetrazin-3-yl)nicotinamide (**7**)

In a round bottom flask **6** (56 mg, 0.26 mmol, 1 eq) HBTU (107 mg, 0.28 mmol, 1.1 eq) and HOBt (3.94 mg, 0.03 mmol, 0.1 eq) were dried *in vacuuo* overnight. In a separate flask DMT-protected propargylamino modified deoxyuridine (150 mg, 0.26 mmol, 1 eq) was also dried overnight *in vacuo*. The mixture of **6**, HBTU and HOBt was dissolved in 5 mL of dry DMF and was stirred for 15 min. Subsequently 60 µL DIPEA 0.35 mmol, 1.35 eq) were added and the solution was stirred for another 15 min. The DMT-protected propargylamino-deoxyuridine was also dissolved in 2 mL of dry DMF and added to the first solution, and the resulting mixture was stirred overnight at room temperature. The solvent was removed under reduced pressure at 40 °C and the residue was purified by column chromatography (SiO_2, CH_2Cl_2/MeOH 30/1v/v +0.1% DIPEA) to furnish **7** in 65% yield.

T.l.c.: R_f = 0.3 (CH_2Cl_2/MeOH 15/1 v/v).

^{1}HNMR (300 MHz, DMSO-d_6): δ = 11.70 (1H, s), 9.38 (1H, t, J = 5.0), 9.28 (1H, m), 8.61 (1H, d, J = 8.2), 8.49 (1H, d, J = 8.2), 7.94 (1H, s), 7.30 (9H, m), 6.89 (4H, m), 6.10 (1H, t, J = 6.3), 5.34 (1H, d, J = 4.4), 4.27 (1H, m), 4.22 (2H, d, J = 5.5), 3.92 (1H, m), 3.72 (6H, s), 3.25 (2H, m), 3.06 (3H, s), 2.25 (2H, m).

^{13}C-NMR (100 MHz, DMSO-d_6) = 167.75, 163.9, 162.9, 161.7, 158.1, 152.35, 149.4, 149.3, 144.8, 143.5, 136.85, 135.7, 135.2, 131.2, 129.8, 129.7, 128.4, 127.0, 128.58, 123.45, 113.3, 98.3, 89.1, 85.9, 85.1, 74.5, 70.5, 63.8, 55.1, 53.6, 45.8, 41.9, 29.5, 21.1, 18.1, 16.8, 12.6, 8.7.

HRMS (ESI) $[M+H]^+$ calcd. for: $C_{42}H_{39}N_8O_8^+$: 783.2884, found: 783.2885.

(2R,3S,5R)-2-((bis(4-methoxyphenyl)(phenyl)methoxy)methyl)-5-(5-(3-(6-(6-methyl-1,2,4,5-tetrazin-3-yl)nicotinamido)prop-1-yn-1-yl)-2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)tetrahydrofuran-3-yl (2-cyanoethyl) diisopropylphosphoramidite (**8**)

In a round bottom flask under argon atmosphere 80 mg **7** (80 mg, 102 µmol, 1 eq) was dissolved in 2 mL abs. CH_2Cl_2 and 36.7 µL DIPEA (200 µmol, 2 eq) was added. To this solution 2-cyanoethyl-*N,N*-diisopropylchloro phosphoramidite (34.2 µL, 153 µmol, 1.5 eq) was added and the reaction was stirred for 1.5 h at room temperature. The reaction mixture was directly taken onto the chromatography column to elute the product very fast (SiO_2, CH_2Cl_2/Aceton 3/2 v/v + 0.1% DIPEA). Evaporation of the solvent *in vacuo* and the resulting pink solid was lyophilized with benzene to obtain **8** in 89% yield.

T.l.c.: R_f = 0.8 (CH_2Cl_2/Acetone 3/2 v/v).

^{31}PNMR (101 MHz, DMSO-d_6); δ = 150.07, 149.67. HRMS (ESI) $[M+H]^+$ calcd. for: $C_{51}H_{56}N_{10}O_9P^+$: 983.3964 found: 983.3962.

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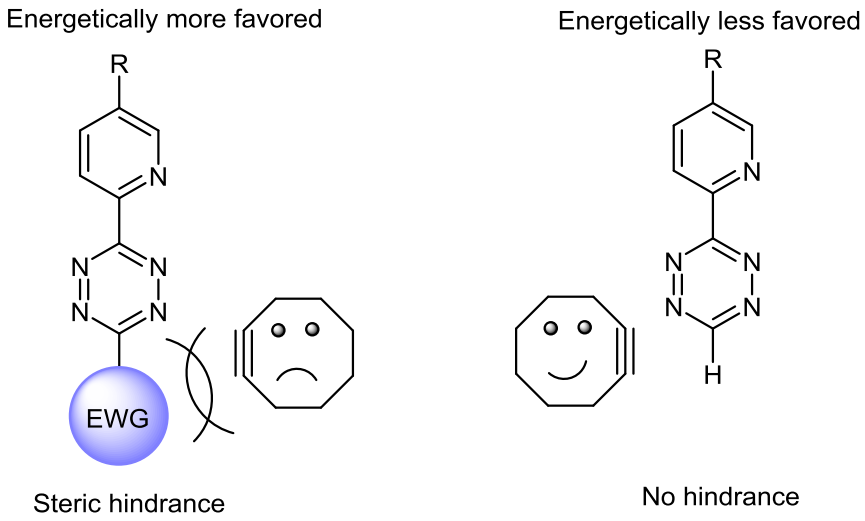

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