Supporting Information for:

Stereochemical promiscuity in artificial transcriptional activators

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Complete citation for references 1b and 1c in the Communication:

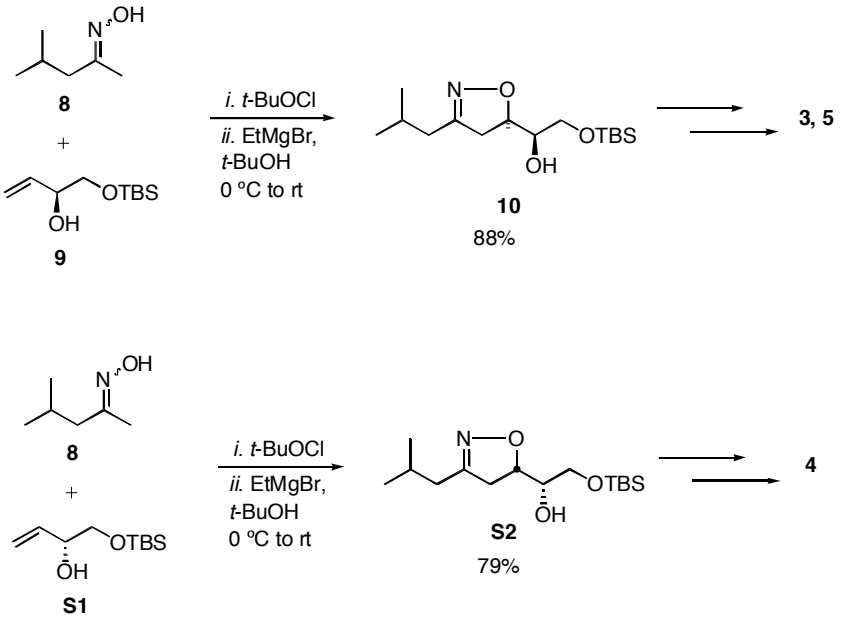
1b: Gavin, A.C.; Bosche, M.; Krause, R.; Grandi, P.; Marzioch, M.; Bauer, A.; Schultz, J.; Rick, J.M.; Michon, A.M.; Cruciat, C.M.; Remor, M.; Hofert, C.; Schelder, M.; Brajenovic, M.; Ruffner, H.; Merino, A.; Klein, K.; Hudak, M.; Dickson, D.; Rudi, T.; Gnau, V.; Bauch, A.; Bastuck, S.; Huhse, B.; Leutwein, C.; Heurtier, M.A.; Copley, R. R.; Edelmann, A.; Querfurth, E.; Rybin, V.; Drewes, G.; Raida, M.; Bouwmeester, T.; Bork, P.; Seraphin, B.; Kuster, B.; Neubauer, G.; Superti-Furga, G. *Nature* **2002**, *415*, 141-7.

1c: Ho, Y.; Gruhler, A.; Heilbut, A.; Bader, G.D.; Moore, L.; Adams, S.L.; Millar, A.; Taylor, P.; Bennett, K.; Boutilier, K.; Yang, L.; Wolting, C.; Donaldson, I.; Schandorff, S.; Shewnarane, J.; Vo, M.; Taggart, J.; Goudreault, Ml; Muskat, B.; Alfarano, C.; Dewar, D.; Lin, Z.; Michalickova, K.; Willems, A.R.; Sassi, H.; Nielsen, P. A.; Rasmussen, K.J.; Andersen, J.R.; Johansen, L.E.; Hansen, L.H.; Jespersen, H.; Podtelejnikov, A.; Nielsen, E.; Crawford, J.; Poulsen, V.; Sorensen, B.D.; Matthiesen, J.; Hendrickson, R.C.; Gleeson, F.; Pawson, T.; Moran, M.F.; Durocher, D.; Mann, M.; Hogue, C.W.; Figeys, D.; Tyers, M. *Nature* **2002** *415*, 180-3.

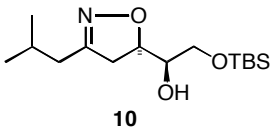
General. Unless otherwise noted, starting materials were obtained from commercial suppliers and used without further purification. CH_2Cl_2, THF, CH_3CN and toluene were dried by passage through activated alumina columns and degassed by stirring under a dry N_2 atmosphere.[1] BF_3•OEt_2 and Et_3N were distilled from CaH_2, MeOH was distilled from sodium metal, and *t*-BuOH was distilled from $MgSO_4$. All reactions involving air- or moisture-sensitive reagents were performed under a dry N_2 atmosphere. Purification by column chromatography was carried out with E. Merck Silica Gel 60 (230-400 mesh) according to the procedure of Still, Kahn, and Mitra.[2] ^1H and ^{13}C NMR spectra were recorded in $CDCl_3$ at 500 MHz and 125 MHz, respectively, unless otherwise specified. IR spectra were measured as thin films on NaCl plates. Reverse-phase HPLC purification was performed on a Varian ProStar 210 equipped with Rainin Dynamax UV-D II detector using a C18 (8 x 100 mm) Radial-PakTM cartridge using a gradient mixture of 20 mM NH_4OAc (pH = 6.9) and MeOH (λ = 254 nm) unless otherwise specified. UV-vis spectra were measured in MeOH. In order to determine the concentration of all methotrexate conjugates (**3-7**), the characteristic UV-vis absorptions of methotrexate at λ_{max} = 257, 302, and 370 nm with extinction coefficients of 23,000, 22,000, and 7,100 $M^{-1}cm^{-1}$, respectively, was used.[3] Once the concentration was determined, the sample was aliquoted, lyophilized, and stored at –78 °C. The in vitro transcription assays were carried out as previously described.[4] The buffer used for transcription assays contains 5 mM $MgCl_2$, 400 mM of each NTP, 10 μg of salmon sperm carrier DNA, 10 mM HEPES (pH 7.9), 50 mM KCl, 0.1 mM EDTA, 0.25 mM DTT, and 10% glycerol. Full fluorescence spectra were run on all methotrexate conjugates to insure no spectral overlap with the molecular beacon fluorophores. Compounds that do not appear in the text are numbered **S1**-**S13.**

3-Methylbutyraldehyde oxime (**8**) was prepared in accordance with standard protocols from 3-methyl-butyraldehyde.[5,6] Allylic alcohols **9** and **S1** were prepared in two steps from (*R*)-glycidol or (*S*)-glycidol, respectively.[7,8] Methotrexate hydrazide was prepared according to literature procedures.[4,9,10] Compound **7** was prepared as previously described.[4] Spectroscopic data on the purified products was consistent with reported values for those compounds.

Synthesis of targets 3-5.

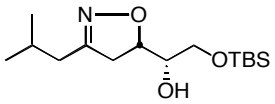


Scheme 1



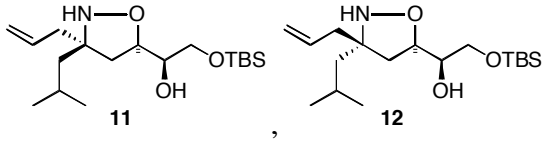
2-(*tert*-Butyl-dimethyl-silanyloxy)-(1*R*)-1-[(5*R*)-3-isobutyl-4,5-dihydro-isoxazol-5-yl]-ethanol (10): To a solution of oxime **8** (780 mg, 7.6 mmol, 1.0 eq) in toluene (38 mL), cooled in a dry ice-acetone bath was added *t*-BuOCl (0.90 mL, 7.6 mmol, 1.0 eq) over 20 min. The resulting mixture was stirred 2 h with continued cooling at which time TLC analysis indicated complete conversion to the hydroximinoyl chloride. In a separate flask, chiral allylic alcohol **9** (2.0 g, 9.9 mmol, 1.3 eq) was dissolved in toluene (99 mL) and cooled in an ice-H_2O bath. To this solution was added *t*-BuOH (25 mmol, 3.3 eq) followed by dropwise addition of EtMgBr (7.6 mL of a 2.0 M solution in Et_2O, 23 mmol, 3.0 eq) and the solution stirred 1 h with continued cooling. The solution of hydroximinoyl chloride was then transferred via canula to the allylic alcohol solution and the mixture allowed to slowly warm to ambient temperature and stirred for 15 h. Sat. aq. NH_4Cl (10 mL) was added to the reaction mixture followed by further dilution with H_2O.

The organic and aqueous layers were separated and the aqueous extracted with CH_2Cl_2 (3 x 20 mL). The combined organic extracts were washed with brine (1 x 20 mL), dried over $MgSO_4$, filtered, and concentrated. Purification by flash chromatrography (1:1 hexanes/EtOAc) yielded 2.0 g of isoxazoline **10** as a clear solid in 88% yield as a single stereoisomer. IR: 3400, 2955, 2858, 1463, 1255 cm^{-1}; ^1H NMR: δ -5.26, (s, 6H), 0.90 (s, 9H), 0.96 (d, 3H, J = 6.6), 0.97 (d, 3H, J = 6.8), 1.90-1.93 (m, 1H), 2.23 (d, 2H, J = 7.3), 2.97 (d, 2H, J = 8.8), 3.58-3.62 (m, 1H), 3.65-3.73 (m, 2H), 4.62-4.67 (m, 1H); ^{13}C NMR (100 MHz): δ -5.26, 18.44, 22.54, 22.74, 26.04, 26.43, 36.66, 39.56, 64.19, 73.26, 79.22, 159.14; HRMS (ESI) calcd for $[C_{15}H_{31}NO_3Si + Na]^+$: 324.1971, found: 324.1985; $[\alpha]_D^{25}$ = -56.17 (*c* 0.84, $CHCl_3$).



S2

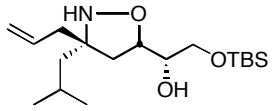
2-(*tert*-Butyl-dimethyl-silanyloxy)-(1*S*)-1-[(5*S*)-3-isobutyl-4,5-dihydro-isoxazol-5-yl]-ethanol (S2): Isoxazoline **S2** was prepared using a procedure[12] analogous to that used for the preparation of **10** except 190 mg (1.9 mmol) of **8** and 500 mg (2.5 mmol) of **S1** were used. Purification by flash chromatography (2:3 hexanes/EtOAc) yielded 410 mg of isoxazoline **S2** as a colorless oil in 79% yield. Spectral data was identical to that of **10**. $[\alpha]_D^{25}$ = +59.50 (*c* 0.87, $CHCl_3$).



11 , **12**

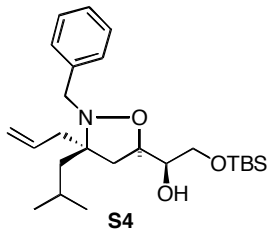
11 and 12: To a solution of isoxazoline **10** (1.5 g, 5.0 mmol, 1.0 eq) in 50 mL toluene cooled in a dry ice-acetone bath was added distilled BF_3•OEt_2 (1.9 mL, 15 mmol, 3.0 eq) and the resultant mixture was stirred with continued cooling for 30 min. Allylmagnesium chloride (15 mL of a 2.0 M solution in THF, 30 mmol, 6.0 eq) was added dropwise over 10 min. The reaction mixture was allowed to stir with continued cooling until the reaction was complete by TLC analysis (6 h). H_2O (5 mL) was added and the mixture stirred for 20 min. H_2O (20 mL) was added and the aqueous and organic layers were separated. The aqueous layer was extracted with EtOAc (3 x 20 mL) and the combined organic extracts were washed with H_2O (1 x 20 mL) and brine (1 x 20 mL), dried over $MgSO_4$, filtered and concentrated *in vacuo*. A diastereomeric ratio of 5:1 was determined by crude ^1H NMR. The major diastereomer (**11**) (1.0 g) and the minor diastereomer (**12**) (200 mg) were each isolated (both as colorless oils) following purification by flash chromatography (1:4 hexanes/EtOAc). The combined yield of diastereomers was 71%. **(1*R*)-1-[(3*S*, 5*R*)-3-Allyl-3-isobutyl-isoxazolidin-5-yl]-2-(*tert*-butyl-dimethyl-silanyloxy)-ethanol (11)**: IR: 3362, 2917, 2849, 1812, 1700, 1076 cm^{-1}; ^1H NMR: δ 0.050 (s, 3H), 0.054 (s, 3H) 0.88 (s, 9H), 0.92 (d, 3H, J = 2.9), 0.93 (d, 3H, J = 2.9), 1.40 (dd, 1H, J = 6.8, 14.2), 1.46 (dd, 1H, J = 6.3, 14.2), 1.77-1.86 (m, 1H), 1.93 (dd, 1H, J = 6.8, 12.2), 2.19 (dd, 2H, J = 7.8, 14.2), 2.33 (dd, 1H, J = 6.8, 14.2), 2.51 (bs, 1H), 3.57-3.63 (m, 2H), 3.66-3.72 (m, 1H), 4.02 (bs, 1H), 5.06-5.12 (m, 2H), 5.34 (bs, 1H), 5.80-

5.88 (m, 1H); ^{13}C NMR (100 MHz): δ -5.52, -5.51, 18.12, 23.91, 24.18, 25.76, 39.98, 40.24, 42.63, 64.25, 64.66, 67.06, 74.13, 117.4, 134.5; HRMS (ESI) calcd for $[C_{18}H_{37}NO_3Si + Na]^+$: 366.2440, found: 366.2435; $[\alpha]_D^{24}$ = -22.03 (*c* 0.81, $CHCl_3$).
(1*R*)-1-[(3*S*, 5*R*)-3-Allyl-3-isobutyl-isoxazolidin-5-yl]-2-(*tert*-butyl-dimethyl-silanyloxy)-ethanol (12): IR: 3400, 2929, 147, 1253, 1113, 836 cm^{-1}; ^{1}H NMR (400 MHz): δ 0.05 (s, 6H), 0.88 (s, 9H), 0.93 (d, 3H, J = 6.8), 0.96 (d, 3H, J = 6.4), 1.39 (dd, 1H, J = 5.9, 14.2), 1.47 (dd, 1H, J = 6.1, 14.4), 1.76-1.84 (m, 1H), 1.99-2.07 (m, 2H), 2.24 (dd, 1H, J = 7.8, 14.2), 2.44 (dd, 1H, J = 6.8, 14.2), 2.49 (bs, 1H), 3.60-3.69 (m, 3H), 4.02-4.06 (m, 1H), 5.10-5.13 (m, 2H), 5.78-5.87 (m, 1H); ^{13}C NMR (100 MHz): δ -5.43, 18.24, 24.33, 24.40, 25.85, 39.20, 40.22, 41.89, 44.49, 63.94, 64.61, 73.40, 118.8, 133.6; HRMS (ESI) calcd for $[C_{18}H_{37}NO_3Si + Na]^+$: 366.2440, found: 366.2446.



S3

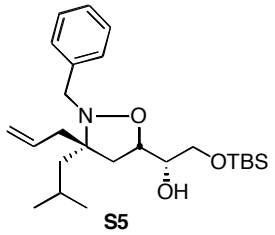
(1*S*)-1-[(3*R*, 5*S*)-3-Allyl-3-isobutyl-isoxazolidin-5-yl]-2-(*tert*-butyl-dimethyl-silanyloxy)-ethanol (S3): Isoxazolidine **S3** was prepared by the same procedure used for the preparation of **11** and **12** except 390 mg (1.4 mmol) of isoxazoline **S2** was used as the starting material. Purification of the crude oil by flash chromatography (1:4 hexanes/EtOAc) yielded 300 mg of **S3** as a colorless oil. Spectroscopic data was identical to that obtained for **11**; $[\alpha]_D^{24}$ = +25.70 (*c* 1.42, $CHCl_3$).



S4

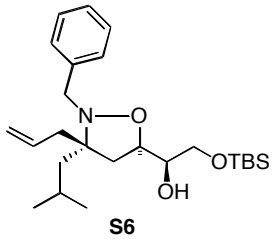
(1*R*)-[(3*S*, 5*R*)-3-Allyl-2-benzyl-3-isobutyl-isoxazolidin-5-yl]-2-(*tert*-butyl-dimethyl-silanyloxy)-ethanol (S4): To a solution of isoxazolidine **11** (210 mg, 0.61 mmol, 1.0 eq) in DMF (3.0 mL) was added *i*Pr_2NEt (0.31 mL, 1.8 mmol, 3.0 eq) and BnBr (0.48 mL, 3.7 mmol, 6.0 eq). The reaction mixture was irradiated in a 1000 W microwave (6 x 20 s) @ 20% power with mixing between each interval. Upon cooling to ambient temperature the solution was diluted with H_2O (3 mL) and extracted with Et_2O (3 x 5 mL). The combined organic extracts were washed with H_2O (1 x 5 mL) and brine (1 x 5 mL), dried over Na_2SO_4, filtered, and concentrated *in vacuo*. Purification of the crude mixture by flash chromatography (95:5 hexanes/EtOAc) yielded 200 mg of **S3** in 77% yield as a clear oil. IR: 3326, 2917, 1803, 1720, 1457, 1081 cm^{-1}; ^{1}H NMR: δ -0.01 (s, 3H), -0.02 (s, 3H), 0.85 (s, 9H), 0.96 (d, 3H, J = 2.9), 0.98 (d, 3H, J = 2.9), 1.39 (dd, 1H, J = 7.3, 14.6), 1.63 (dd, 1H, J = 4.6, 14.4), 1.87-1.95 (m, 1H), 2.26 (m, 2H), 2.32 (dd, 1H, 8.8, 12.2), 2.44 (dd, 1H, J = 7.1, 13.9), 3.13 (bs, 1H), 3.41 (dd, 1H, J = 6.3, 8.8), 3.46-3.53 (m, 2H), 3.80 (d, 1H, J = 14.2), 3.85 (d, 1H, J = 14.2), 4.03-4.06 (m, 1H), 5.09-5.12

(m, 2H), 5.91-5.99 (m, 1H), 7.21-7.24 (m, 1H), 7.28-7.34 (m, 4H); ^{13}C NMR (100 MHz): δ -5.42, 18.31, 24.10, 24.33, 25.33, 25.91, 38.53, 39.56, 44.08, 53.67, 64.34, 68.62, 74.92, 117.62, 126.93, 128.27, 128.28, 135.19, 138.61; HRMS (ESI) calcd for $[C_{25}H_{43}NO_3Si]^+$: 434.3090, found: 434.3089; $[\alpha]_D^{24}$ = -15.61 (*c* 1.95, $CHCl_3$).



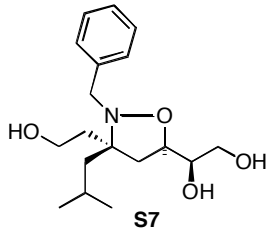
S5

(1*S*)-[(3*R*, 5*S*)-3-Allyl-2-benzyl-3-isobutyl-isoxazolidin-5-yl]-2-(*tert*-butyl-dimethyl-silanyloxy)-ethanol (S5): Isoxazolidine **S5** was prepared by the procedure used for the preparation of **S4** except 460 mg (1.3 mmol) of isoxazolidine **S3** was used as the starting material. Purification by flash chromatography (95:5 hexanes/EtOAc) gave 470 mg (80% yield) of product isolated as a clear oil. Spectroscopic data was identical to the enantiomer **S4**. $[\alpha]_D^{24}$ = +11.32 (*c* 1.12, $CHCl_3$).



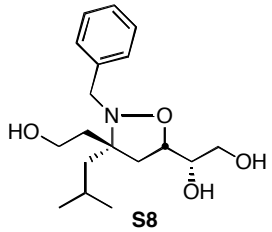
S6

(1*R*)-[(3*R*, 5*R*)-3-Allyl-2-benzyl-3-isobutyl-isoxazolidin-5-yl]-2-(*tert*-butyl-dimethyl-silanyloxy)-ethanol (S6): Isoxazolidine **S6** was prepared using the procedure for the preparation of **S4** from 200 mg (0.58 mmol) of **12**. Purification by flash chromatography (95:5 hexanes/EtOAc) gave **S6** in 81% yield (200 mg) as an oil. IR: 3430, 2928, 1463, 1253, 1115, 837 cm^{-1}; ^{1}H NMR: δ -0.02 (s, 6H), 0.84 (s, 9H), 0.98 (d, 3H, J = 8.3), 1.00 (d, 3H, J = 8.3), 1.46 (dd, 1H, J = 4.4, 13.2), 1.78-1.86 (m, 1H), 2.21-2.36 (m, 3H), 2.47 (dd, 1H, J = 6.8, 13.7), 3.42 (dd, 1H, J = 8.3, 11.7), 3.49-3.53 (m, 2H), 3.72 (d, 1H, J = 13.2), 3.84 (d, 1H, J = 13.2), 4.08 (bs, 1H), 5.10-5.13 (m, 2H), 5.89-5.98 (m, 1H), 7.20-7.23 (m, 1H), 7.27-7.33 (m, 4H); ^{13}C NMR: δ -5.46, 18.28, 24.61, 24.94, 25.03, 25.90, 39.20, 40.17, 40.25, 41.54, 53.60, 64.34, 74.34, 117.87, 117.92, 126.92, 128.24, 128.48, 134.35; HRMS (ESI) calcd for $[C_{25}H_{43}NO_3Si]^+$: 434.3090, found: 434.3086; $[\alpha]_D^{25}$ = -1.50 (*c* 0.20, MeOH).



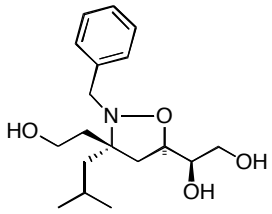
S7

(1*R*)-1-[(3*S*, 5*R*)-2-Benzyl-3-(2-hydroxy-ethyl)-3-isobutyl-isoxazolidin-5-yl]-ethane-1,2-diol (S7): To a solution of isoxazolidine **S4** (0.15 g, 0.34 mmol, 1.0 eq) in *t*-BuOH (2.5 mL), THF (0.67 mL), and H_2O (0.17 mL) was added NMO (47 mg, 0.40 mmol, 1.2 eq) followed by OsO_4 (0.34 ml of a 2.5 wt% solution in *t*-BuOH, 0.03 mmol, 0.10 eq). The reaction mixture was stirred at ambient temperature until complete by TLC analysis (5 h). The mixture was cooled in an ice-H_2O bath, Na_2SO_3 (20 mg) was added, and the mixture stirred 1 h. The mixture was diluted with H_2O (10 mL) and extracted with EtOAc (3 x 10 mL). The combined organic extracts were dried over Na_2SO_4, filtered and concentrated *in vacuo*. The crude diol was taken up in 1.9 mL CH_3CN and 1.9 mL H_2O and cooled in an ice-H_2O bath. Sodium periodate (0.10 g, .45 mmol, 1.2 eq) was added and the reaction mixture stirred at ambient temperature until complete by TLC analysis (2 h). The reaction mixture was diluted with H_2O (10 mL) and extracted with Et_2O (3 x 10 mL). The combined organic extracts were washed with H_2O (1 x 10 mL) and brine (1 x 10 mL), dried over Na_2SO_4, filtered and concentrated *in vacuo*. The crude aldehyde thus obtained was dissolved in 3.7 mL MeOH and cooled in an ice-H_2O bath prior to addition of $NaBH_4$ (21 mg, 0.56 mmol, 1.5 eq). Upon completion as noted by TLC analysis (1h), H_2O (5 mL) was added and the reaction extracted with EtOAc (3 x 10 mL). The combined organic extracts were dried over Na_2SO_4, filtered and concentrated *in vacuo*. The crude material was passed through a plug of SiO_2 to remove baseline impurities (7:3 hexanes/EtOAc). To a portion of the product (0.20 mmol, 1.0 eq) in THF (1.0 mL) cooled in an ice-H_2O bath was added TBAF (0.41 mL of a 1 M solution in THF, 0.41 mmol, 2.0 eq). The reaction mixture was allowed to stir at ambient temperature until complete by TLC analysis (2 h). The mixture was then diluted with H_2O (10 mL) and extracted with EtOAc (5 x 15 mL). The combined organic extracts were dried over Na_2SO_4, filtered, and concentrated *in vacuo*. Purification by flash chromatography (93:7 CH_2Cl_2: MeOH) provided 83 mg of **S7** in 59% yield from compound **S4** as a colorless oil. IR: 3349, 2952, 1453, 1063 cm^{-1}; ^1H NMR (400 MHz): δ 0.99 (d, 3H, J = 6.6), 1.01 (d, 3H, J = 6.6), 1.44 (dd, 1H, J = 8.1, 13.9), 1.71-1.82 (m, 2H), 1.83-2.00 (m, 2H), 2.22-2.34 (m, 2H), 3.50-3.61 (m, 3H), 3.77-3.85 (m, 3H), 4.01 (d, 1H, J – 13.6), 4.10-4.19 (m, 1H), 7.11-7.38 (m, 5H); ^{13}C NMR (100 MHz): δ 23.55, 24.77, 25.16, 35.30, 39.93, 42.82, 54.79, 59.61, 63.98, 64.94, 70.45, 73.53, 127.31, 128.44, 128.56, 137.63; HRMS (ESI) calcd for $[C_{18}H_{29}NO_4 + Na]^+$: 346.1994, found: 346.1993; $[\alpha]_D^{27}$ = + 0.09 (*c* 0.29, $CHCl_3$).



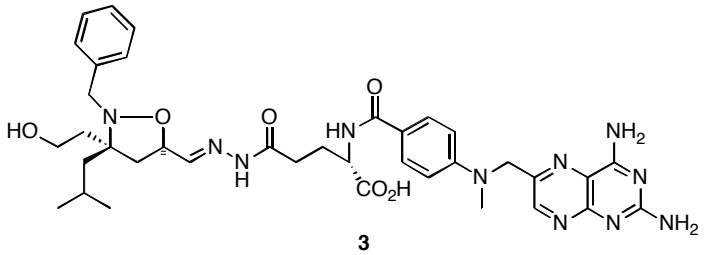
S8

(1*S*)-1-[(3*R*, 5*S*)-2-Benzyl-3-(2-hydroxy-ethyl)-3-isobutyl-isoxazoldin-5-yl]-ethane-1,2-diol (S8): Experimental conditions for the conversion of **S5** (150 mg, 0.34 mmol) to **S8** followed the procedure used for the conversion of **S4** to **S7**. Purification by flash chromatography (93:7 CH_2Cl_2:MeOH) provided 81 mg of **S8** in 75% yield as a colorless oil. Spectroscopic data was identical to that obtained for **S6**. $[\alpha]_D^{27}$ = -0.08 (*c* 0.29, $CHCl_3$).



S9

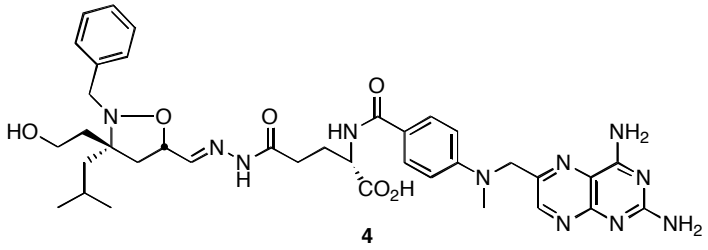
(1*R*)-1-[(3*R*, 5*R*)-2-Benzyl-3-(2-hydroxy-ethyl)-3-isobutyl-isoxazolidin-5-yl]-ethane-1,2-diol (S9): Oxidative cleavage of the allyl functionality on isoxazolidine **S6** was carried out on 200 mg (0.48 mmol) of **S6** using a procedure analogous to the one used for the oxidative cleavage of **S4**. The final TBS deprotection was carried out on 100 mg (0.23 mmol) of the intermediate alcohol following passage of the crude product through a plug of silica (7:3 hexanes/EtOAc). Purification of the crude oil by flash chromatography (92:8 CH_2Cl_2: MeOH) gave 47 mg of **S9** in 64% yield as a clear oil. IR: 3334, 2954, 1456, 1067 cm^{-1}; ^1H NMR: δ 0.99 (d, 3H, J = 6.3), 1.01 (d, 3H, J = 6.3), 1.57 (dd, 2H, J = 6.8, 13.7), 1.66-1.79 (m, 2H), 2.10 (m, 1H), 2.28 (dd, 1H, J = 5.9, 12.7), 2.38 (dd, 1H, J = 8.8, 12.7), 3.52 (dd, 1H, J = 5.1, 11.5), 3.61 (dd, 1H, J = 3.9, 11.7), 3.67 (m, 1H), 3.75 (d, 1H, J = 13.7), 3.82-3.91 (m, 2H), 4.01 (d, 1H, J = 13.2), 4.27-4.31 (m, 1H), 7.25-7.35 (m, 5H); ^{13}C NMR (100 MHz): 24.23, 25.06, 25.00, 35.74, 38.76, 41.40, 53.30, 59.32, 63.90, 72.97, 127.74, 128.62, 128.82, 137.21; HRMS (ESI) calcd for $[C_{18}H_{29}NO_4 + Na]^+$: 346.1994, found: 346.1999; $[\alpha]_D^{25}$ = +3.21 (*c* 0.20, MeOH).



3

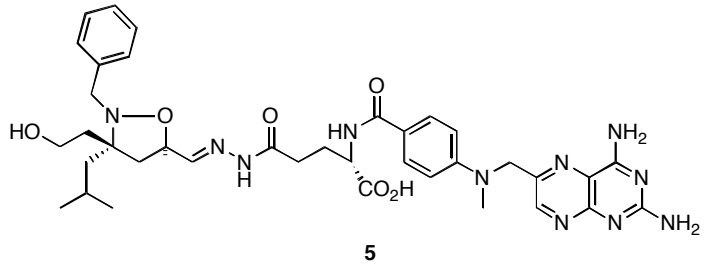
4-[(3*S*, 5*R*)-2-Benxyl-3-(2-hydroxy-ethyl)-3-isobutyl-isoxazolidin-5-ylmethylene-hydrazinocarbonyl]-2-{4-[(2,4-diamino-pteridin-6-ylmethyl)-methyl-amino]-

benzoylamino}-(*S*)-butyric acid (3): To a solution of diol **S7** (9.7 mg, 0.03 mmol, 1.0 eq) in CH_3CN (0.15 mL) and H_2O (0.15 mL) cooled in an ice-H_2O bath was added $NaIO_4$ (5.3 mg, 0.02, 0.80 eq). The solution was slowly warmed to ambient temperature and the formation of aldehyde was monitored by TLC analysis. After 2 h the mixture was diluted with H_2O (2 mL), extracted with Et_2O (3 x 3 mL), and the combined organic extracts washed with brine (2 x 2 mL), dried over Na_2SO_4, filtered, and concentrated *in vacuo.* The crude aldehyde (0.003 mmol, 1.0 eq) in THF (0.16 mL) was added to methotrexate hydrazide (0.003 mmol, 1.0 eq) in DMF (0.16 mL). The reaction was stirred at ambient temperature, shielded from light for 24 h. The mixture was then concentrated to half-volume under high pressure (0.05 mm Hg) and purified by reverse-phase HPLC. Following purification by reverse-phase HPLC, the compound was stored at –78 °C, shielded from light. The purity of **3** was confirmed by analytical reverse-phase HPLC immediately after isolation and again prior to use in any *in vitro* transcription assays. The identity was verified by mass spectral and UV analysis of the isolated conjugate. UV (λ_{max} nm): 257, 297, 373; HRMS (ESI) calcd for $[C_{37}H_{47}N_{11}O_6 + Na]^+$: 764.3608, found: 764.3605.



4

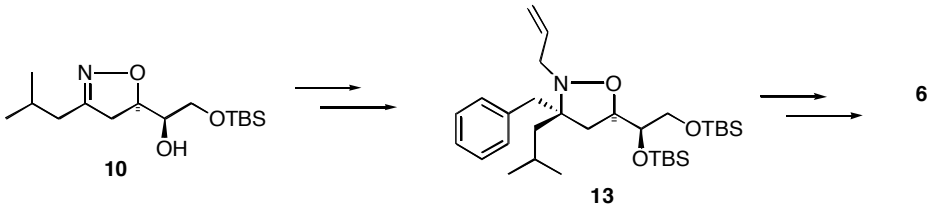
4-[(3*R*, 5*S*)-2-Benxyl-3-(2-hydroxy-ethyl)-3-isobutyl-isoxazolidin-5-ylmethylene-hydrazinocarbonyl]-2-{4-[(2,4-diamino-pteridin-6-ylmethyl)-methyl-amino]-benzoylamino}-(*S*)-butyric acid (4): 4 was prepared, purified, and stored by the same methods as **3**. The identity was verified by mass spectral and UV analysis of the isolated conjugate. UV (λ_{max} nm): 259, 299, 375; HRMS (ESI) calcd for $[C_{37}H_{47}N_{11}O_6 + Na]^+$: 764.3608, found: 764.3608.



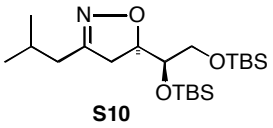
5

4-[(3*R*, 5*R*)-2-Benxyl-3-(2-hydroxy-ethyl)-3-isobutyl-isoxazolidin-5-ylmethylene-hydrazinocarbonyl]-2-{4-[(2,4-diamino-pteridin-6-ylmethyl)-methyl-amino]-benzoylamino}-(*S*)-butyric acid (5): 5 was prepared, purified, and stored by the same methods as **3**. The identity was verified by mass spectral and UV analysis of the isolated conjugate. UV (λ_{max} nm): 258, 298, 378; HRMS (ESI) calcd for $[C_{37}H_{47}N_{11}O_6 + Na]^+$: 764.3608, found: 764.3599.

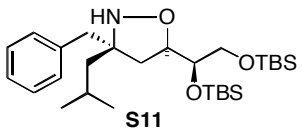
Synthesis of target compound 6.



Scheme 2

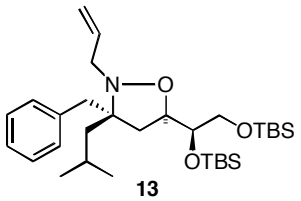


2-(*tert*-Butyl-dimethyl-silanyloxy)-(1*R*)-1-[(5*R*)-(3-isobutyl-4,5-dihydro-isoxazol-5-yl)-ethanol] (S10): To a solution of isoxazoline **10** (360 mg, 1.3 mmol, 1.0 eq) in THF (6.5 mL) cooled in an ice-H_2O bath was added DMAP (16 mg, 0.13 mmol, 0.l0 eq) and Et_3N (0.35 mL, 2.9 mmol, 2.2 eq). TBSOTf (0.67 mL, 2.9 mmol, 2.2 eq) was then added dropwise and the solution slowly warmed to ambient temperature. The reaction was complete in 2 h as indicated by TLC analysis. The mixture was again cooled in an ice-H_2O bath, diluted with sat. NH_4Cl (3 mL), and extracted with Et_2O (3 x 5 mL). The combined organic extracts were washed with brine (1 x 10 mL), dried over Na_2SO_4, filtered, and concentrated *in vacuo*. Purification of the crude product by flash chromatography (95:5 hexanes/EtOAc) yielded 460 mg of isoxazoline **S10** in 85% yield as a clear oil. IR: 3400, 2955, 2858, 1594, 1463, 1255, 1124 cm^{-1}; ^1H NMR: δ 0.01 (s, 3H), 0.02 (s, 3H), 0.04 (s, 3H), 0.05 (s, 3H), 0.83 (s, 9H), 0.84 (s, 9H), 0.90 (d, 3H, J = 6.6), 0.91 (d, 3H, J = 6.6), 1.84-1.87 (m, 1H), 2.13-2.16 (m, 2H), 2.81-2.84 (m, 2H), 3.53-3.56 (m, 1H), 3.61-3.66 (m, 2H), 4.52-4.58 (m, 1H); ^{13}C (100 MHz): δ -5.27, -4.54, -4.20, 18.31, 18.50, 22.64, 22.80, 25.99, 26.11, 26.26, 36.90, 38.78, 64.61, 74.30, 80.52, 158.12; HRMS (ESI) calcd for $[C_{21}H_{45}NO_3Si_2 + Na]^+$: 438.2836, found: 438.2839; $[\alpha]_D^{25}$ = -62.28 (*c* 0.50, $CHCl_3$).



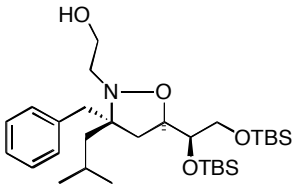
(3*S*, 5*R*)-3-Benzyl-5-[(1*R*)-1,2-bis-(tert-butyl-dimethyl-silanyloxy)-ethyl]-3-isobutyl-isoxazolidine (S11): Benzylmagnesium chloride (4.5 mL of a 2.0 M solution in THF, 9.0 mmol, 10 eq) was added to 370 mg of isoxazoline **S10** (9.0 mmol, 1.0 eq) in 8.9 ml THF in the presence of BF_3•OEt_2 (0.34 mL, 2.7 mmol, 3.0 eq) by using a procedure analogous

to that used for the preparation of **11**. A diastereomeric ratio of 10:1 was determined by crude ^{1}H NMR. Purification by flash chromatography (9:1 hexanes/EtOAc) yielded 320 mg of the major diastereomer in 80% yield as a colorless oil. IR: 2954, 2929, 2858, 1472, 1255 cm^{-1}; ^{1}H NMR: δ 0.03 (s, 3H), 0.03 (s, 3H), 0.05 (s, 3H), 0.06 (s, 3H), 0.85 (s, 9H), 0.87 (s, 9H), 0.91 (d, 3H, J = 6.6), 0.96 (d, 3H, J = 6.6), 1.26 (m, 2H), 1.79-1.84 (m, 1H), 2.02 (m, 1H), 2.18-2.23 (m, 1H), 2.66 (d, 1H, J = 13.9), 2.92 (d, 1H, J = 13.5), 3.53-3.59 (m, 2H), 3.72 (bs, 1H), 4.30 (bs, 1H), 5.20 (bs, 1H), 7.14-7.19 (m, 1H), 7.22-7.24 (m, 4H); ^{13}C NMR: δ -5.17, -4.29, -4.12, 18.18, 18.57, 24.18, 24.62, 24.85, 25.18, 26.13, 40.69, 42.27, 42.98, 64.61, 68.79, 72.31, 78.96, 126.300, 128.04, 130.91, 138.69; HRMS (ESI) calcd for $[C_{28}H_{53}NO_3Si_2 + Na]^+$: 530.3462, found: 530.3464; $[\alpha]_D^{25}$ = -64.98 (*c* 0.26, MeOH).



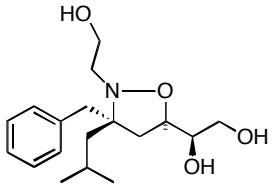
13

(5*R*, 3*S*)-2-Allyl-3-benzyl-5-[(1*R*)-1,2-bis-(*tert*-butyl-dimethyl-silanyloxy)-ethyl]-3-isobutyl-isoxazolidine (13): To a solution of 150 mg of isoxazolidine **S11** (0.29 mmol, 1.0 eq) in DMF (1.4 mL) was added *i*Pr$_2$NEt (110 mg, 0.86 mmol, 3.0 eq) and allylBr (0.20 mL, 2.3 mmol, 8.0 eq). The reaction mixture was irradiated in a 1000 W microwave (6 x 20 s) @ 20 % power with mixing between each interval. A second portion of allylBr was added (.20 mL, 2.3 mmol, 8.0 eq) and the mixture was irradiated in a 1000 W microwave (6 x 20 s) @ 20 % power with mixing between each interval. The solution was diluted with H_2O (3 mL) and extracted with Et_2O (3 x 5 mL). The combined organic extracts were washed with H_2O (1 x 5 mL) and brine (1 x 5 mL), dried over Na_2SO_4, filtered, and concentrated *in vacuo*. Product was isolated as a colorless oil in 65% yield (100 mg) following purification by flash chromatography (97:3 hexanes/EtOAc). IR: 3349, 2929, 1761, 1456, 1255, 1081 cm^{-1}; ^{1}H NMR (400 MHz): δ 0.01 (s, 3H), 0.02 (s, 3H), 0.07 (s, 6H), 0.84 (d, 3H, J = 7.3), 0.86 (s, 9H), 0.87 (s, 9H), 0.94 (d, 3H, J = 6.6), 1.21-1.26 (m, 2H), 1.47-1.52 (m, 1H), 1.77-1.86 (m, 1H), 1.90-2.02 (m, 2H), 2.66 (d, 1H, J = 12.5), 2.86 (d, 1H, J = 13.2), 3.27 (dd, 1H, J = 6.6, 13.9), 3.37 (dd, 1H, J = 5.9, 13.9), 3.54 (dd, 1H, J = 6.6, 11.7), 3.66-3.70 (m, 2H), 4.09 (dt, 1H, J = 5.1, 8.1), 5.06 (dd, 1H, J = 1.5, 10.3), 5.19 (dd, 1H, J = 1.5, 16.8), 5.89-5.99 (m, 1H), 7.17-7.26 (m, 5H); ^{13}C (100 MHz): δ -5.40, -4.58, -4.48, 18.25, 18.36, 23.79, 24.52, 25.27, 25.97, 36.87, 39.57, 42.11, 52.90, 65.39, 69.24, 75.26, 116.03, 125.99, 127.89, 130.76, 136.22, 138.81; HRMS (ESI) calcd for $[C_{31}H_{57}NO_3Si]^+$: 548.3955, found: 548.3953; $[\alpha]_D^{25}$ = -33.17 (*c* 1.19, MeOH).



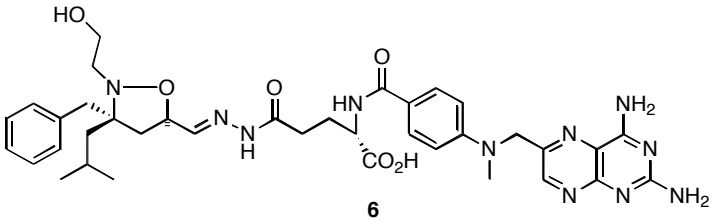
S12

2-{(3*S*, 5*R*)-3-Benzyl-5-[(1*R*)-1,2-bis-(*tert*-butyl-dimethyl-silanyloxy)-ethyl]-3-isobuty-isoxazolidin-2-yl}-ethanol (S12): Oxidative cleavage of the allyl group on **13** to form isoxazolidine **S12** was carried out using a procedure analogous to that used for the preparation of **S7** starting with 95 mg of **13** (0.17 mmol) except that the product was purified and isolated prior to silyl deprotection. Purification by flash chromatography yielded 45 mg of **S12** in 47% yield as a colorless oil. IR: 3369, 2849, 1772, 1472, 1258, 1078 cm^{-1}; ^1H NMR (400 MHz): δ 0.02 (s, 3H), 0.04 (s, 3H), 0.07 (s, 6H), 0.86-0.88 (m, 21H), 0.93 (d, 3H, J = 6.6), 1.21 (dd, 1H, J = 5.9, 13.9), 1.48 (dd, 1H, J = 3.7, 13.9), 1.80-1.89 (m, 1H), 1.97 (dd, 1H, J = 8.8, 12.5), 2.05 (dd, 1H, J = 7.7, 12.1), 2.68 (d, 1H, J = 13.2), 2.74 (t, 1H, J = 5.86), 2.78-2.92 (m, 2H), 3.55 (dd, 1H, J = 7.3, 11.7), 3.62 (dd, 2H, J = 5.1, 11.7), 3.74 (m, 2H), 4.12-4.19 (m, 1H), 7.16-7.27 (m, 5H); ^{13}C NMR: δ -5.46, -5.43, -4.65, -4.48, 18.15, 18.34, 24.52, 25.26, 25.87, 25.93, 37.46, 39.67, 42.13, 51.09, 60.87, 65.224, 69.28, 44.63, 126.18, 127.96, 130.69, 138.37; HRMS (ESI) calcd for $[C_{30}H_{57}NO_4Si_2]^+$: 552.3904, found: 552.3912; $[\alpha]_D^{25}$ = -13.51 (*c* 0.70, MeOH).



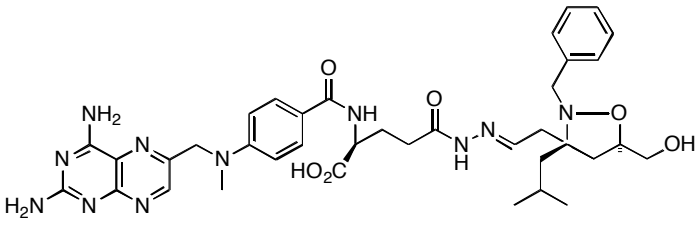
S13

(1*R*)-1-[(3*S*, 5*R*)-3-Benzyl-2-(2-hydroxy-ethyl)-3-isobutyl-isoxazolidin-5-yl]-ethane-1,2-diol (S13): Treatment of 44 mg of isoxazolidine **S12** (0.08 mmol, 1.0 eq) in THF (0.80 mL) cooled in an ice-H_2O bath with TBAF (0.32 mL of a 1 M solution in THF, 0.32 mmol, 4.0 eq) afforded removal of both silyl protecting groups in 2 h by TLC analysis. The solution was diluted with H_2O (5 mL), extracted with EtOAc (3 x 5 mL), dried over Na_2SO_4, filtered, and concentrated *in vacuo*. Purification by flash chromatography (92:8 CH_2Cl_2:MeOH) yielded 25 mg of product in 98% yield as an oil. IR: 3369, 2954, 1456, 1056 cm^{-1}; ^1H NMR: δ 0.87 (d, 3H, J = 6.8), 0.95 (d, 3H, J = 6.3), 1.30 (dd, 1H, J = 6.3, 14.6), 1.58 (dd, 1H, J = 4.4, 14.6), 1.83-1.91 (m, 1H), 2.02-2.07 (m, 1H), 2.17-2.26 (m, 2H), 2.63 (d, 1H, J = 12.7), 2.87 (d, 1H, J = 13.2), 2.93 (m, 2H), 3.59-3.63 (m, 2H), 3.67-3.69 (m, 1H), 3.78-3.86 (m, 2H), 4.09 (td, 1H, J = 3.7, 7.6), 7.15-7.17 (m, 2H), 7.20-7.23 (m, 1H), 7.26-7.29 (m, 2H); ^{13}C NMR (100 MHz): δ 23.55, 24.57, 25.38, 37.42, 39.38, 42.27, 51.09, 61.37, 64.38, 69.77, 74.10, 126.36, 128.14, 130.77, 138.02; HRMS (ESI) calcd for $[C_{18}H_{29}NO_4 + Na]^+$: 346.1994, found: 346.1991; $[\alpha]_D^{25}$ = -19.47 (*c* 0.19, MeOH).



6

4-[(3*S*, 5*R*)-3-Benxyl-2-(2-hydroxy-ethyl)-3-isobutyl-isoxazolidin-5-ylmethylene-hydrazinocarbonyl]-2-{4-[(2,4-diamino-pteridin-6-ylmethyl)-methyl-amino]-benzoylamino}-(*S*)-butyric acid (6): Purification, storage, and purity confirmation was carried out analogously to **3**. The identity was verified by mass spectral and UV analysis of the isolated conjugate. UV (λ_{max} nm): 258, 295, 373; LRMS (ESI) calcd for $[C_{37}H_{47}N_{11}O_6 + H]^+$: 742.8, found: 742.6.



7

2-[4({[6-(Amino-methylcarbaminidoylimino-methyl)-pyrazin-2-ylmethyl]-methyl-amino}-methyl)-benzoylamino]-4-[2-(2-benzyl-(5*RS*)-hydroxymethyl-(3*RS*)-isobutyl-isoxazolidin-3-yl)-ethylidene-hydrazinocarbonyl]-(*S*)-butyric acid (7): Compound **7** was prepared as described previously.[4] The identity was verified by mass spectral analysis of the isolated construct. UV (λ_{max} nm): 260, 299, 375; HRMS (ESI) calcd for $[C_{37}H_{47}N_{11}O_6 + Na]^+$: 764.3608, found: 764.3616.

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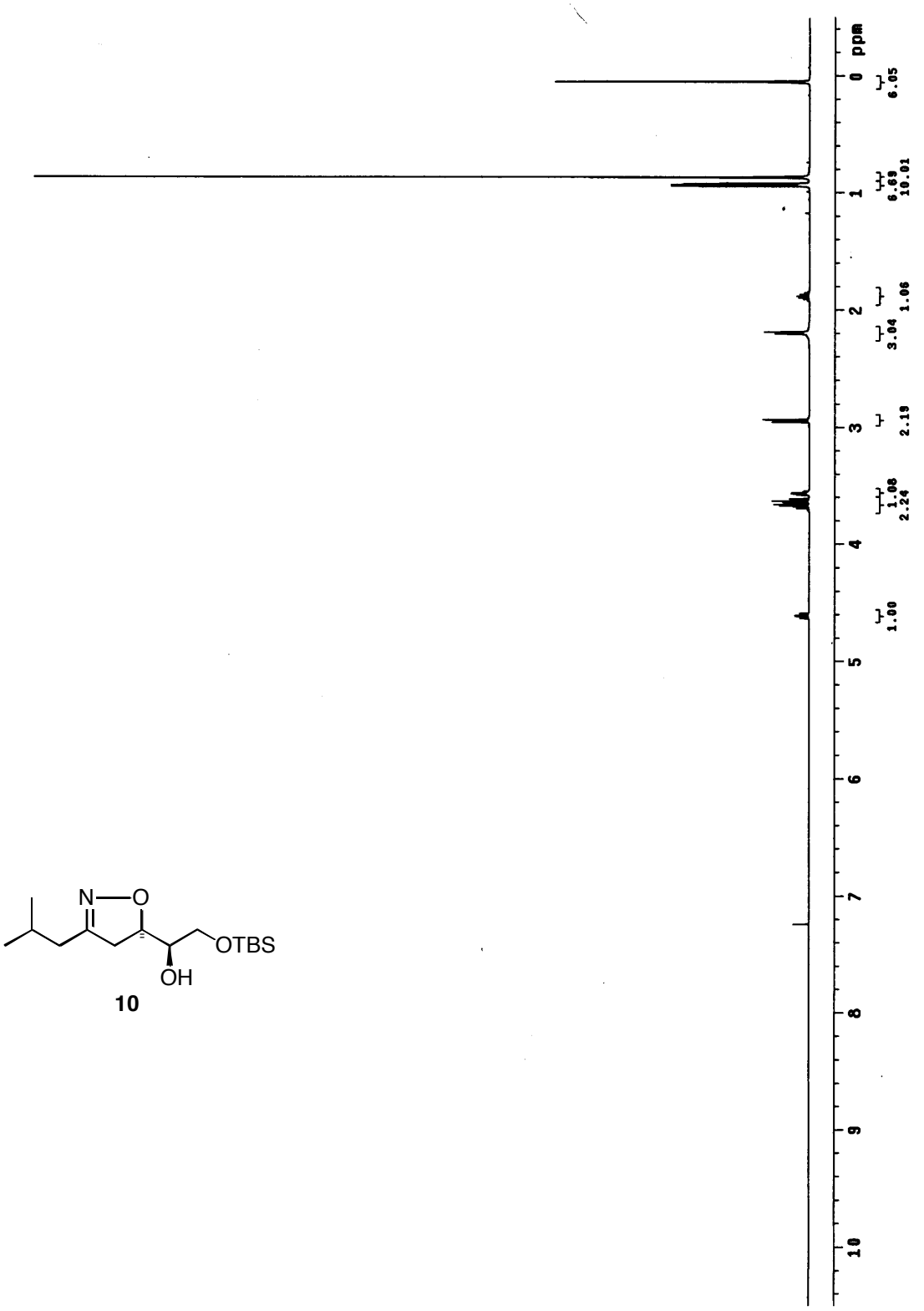
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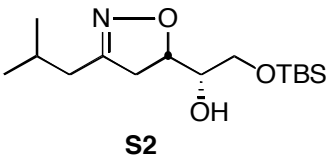
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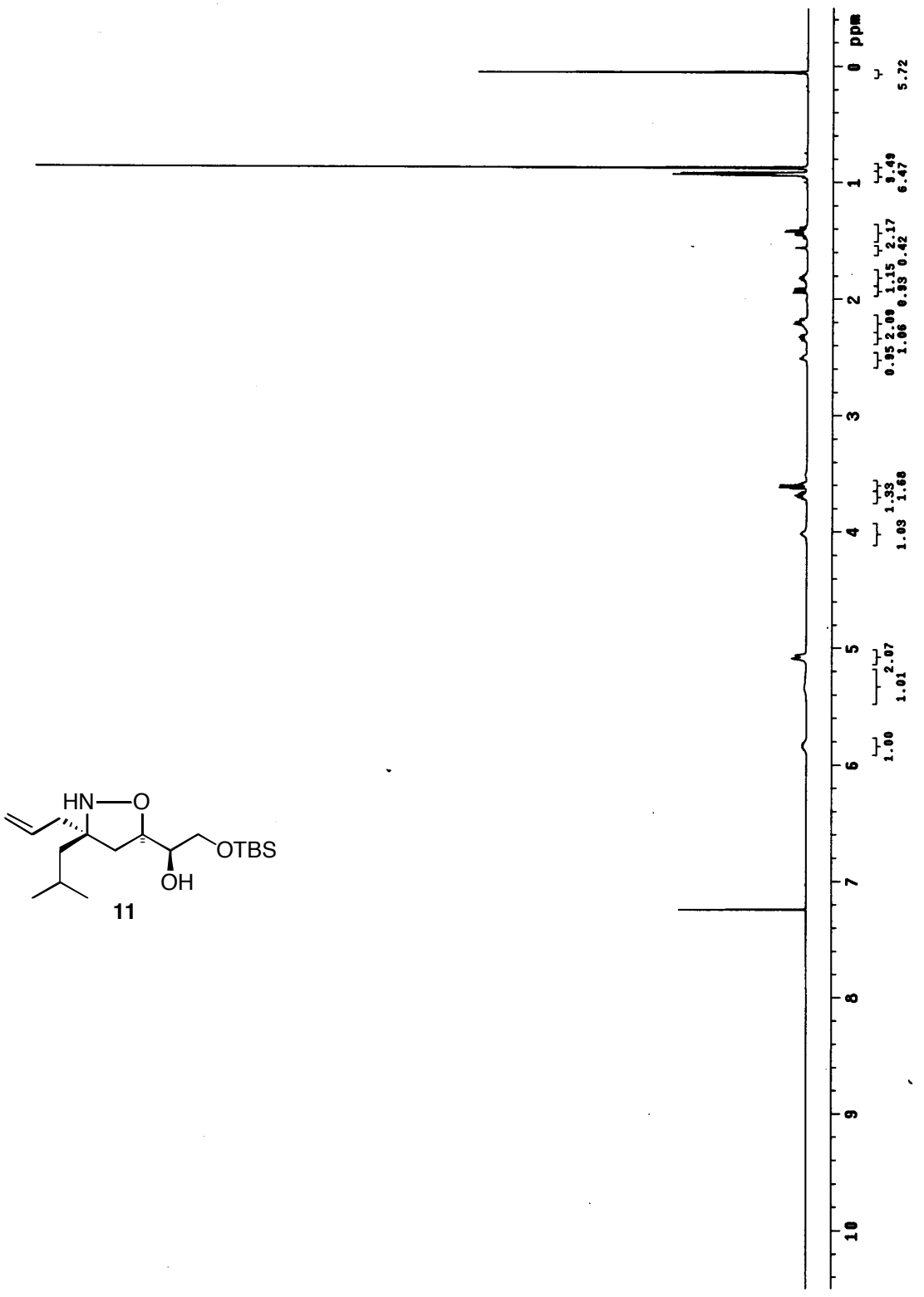
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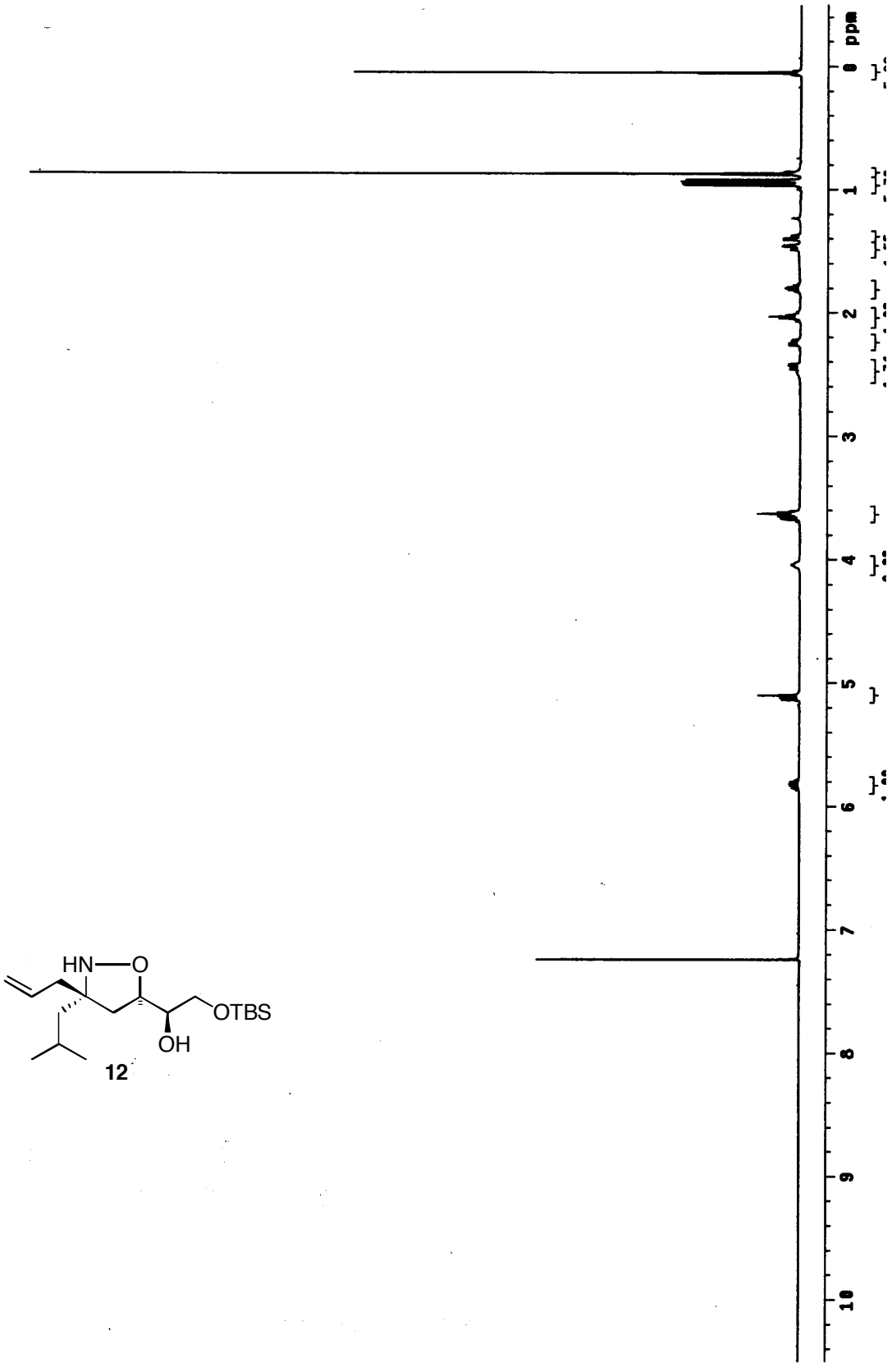
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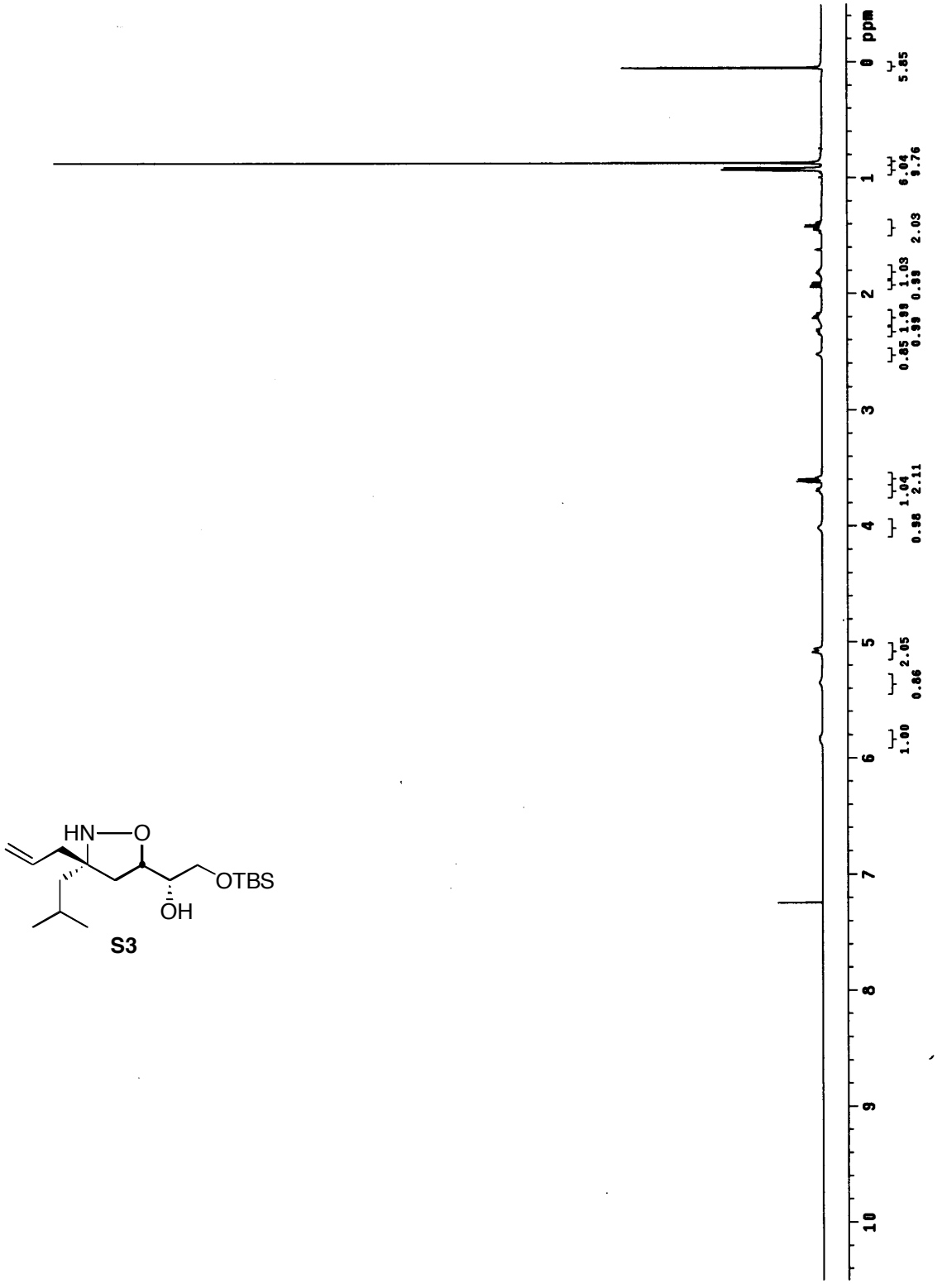
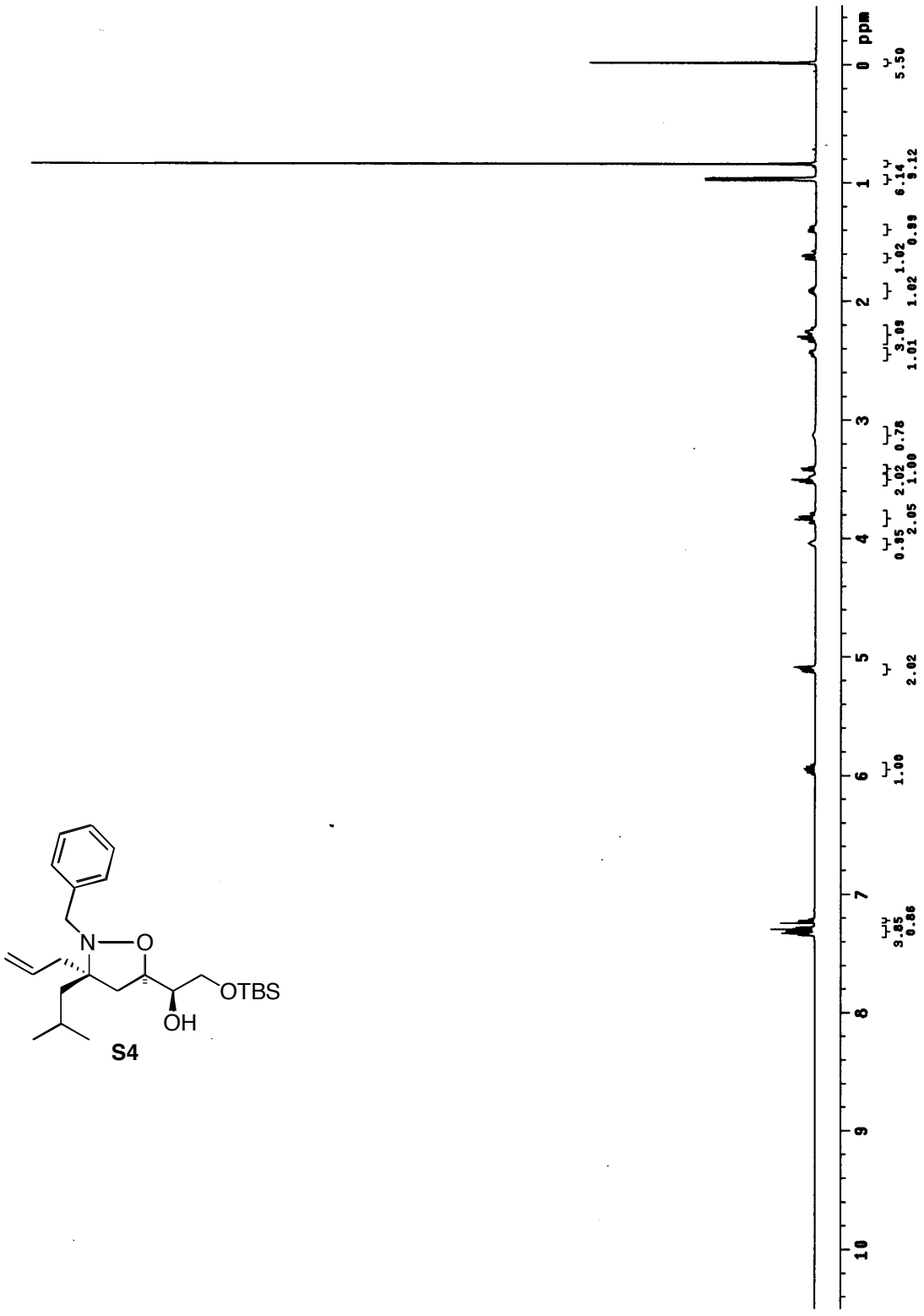
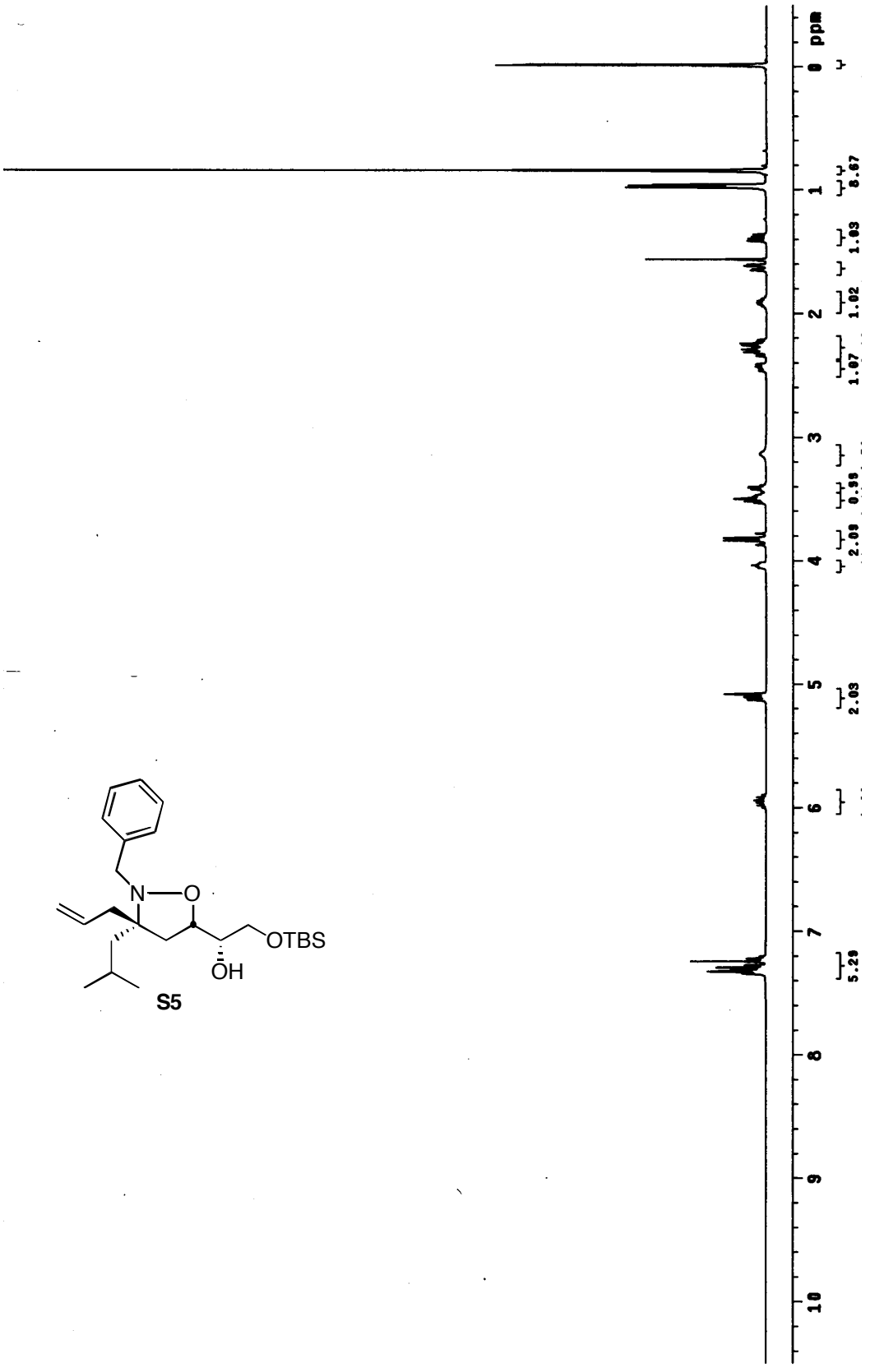
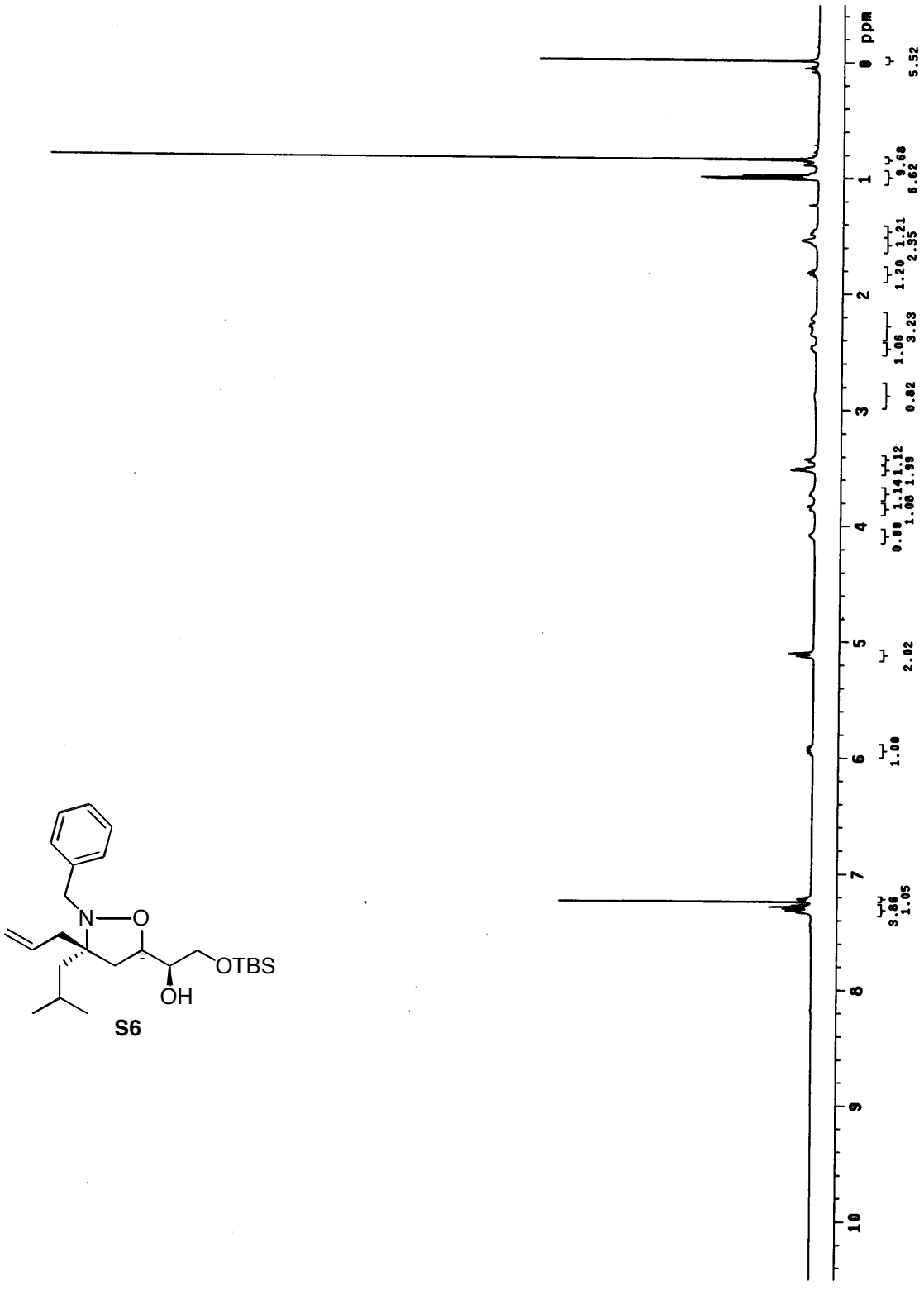
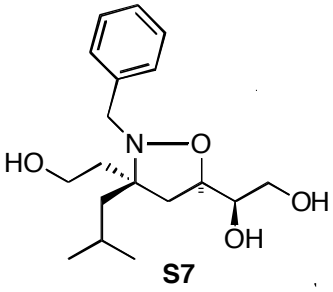
7
8
9
10


HN
O
OTBS
OH
S3
0 ppm
1
2
3
4
5
6
7
8
9
10
5.85
6.04
5.76
2.03
1.03
0.99
0.85
1.00
0.99
1.04
2.11
0.98
2.05
0.86
1.00

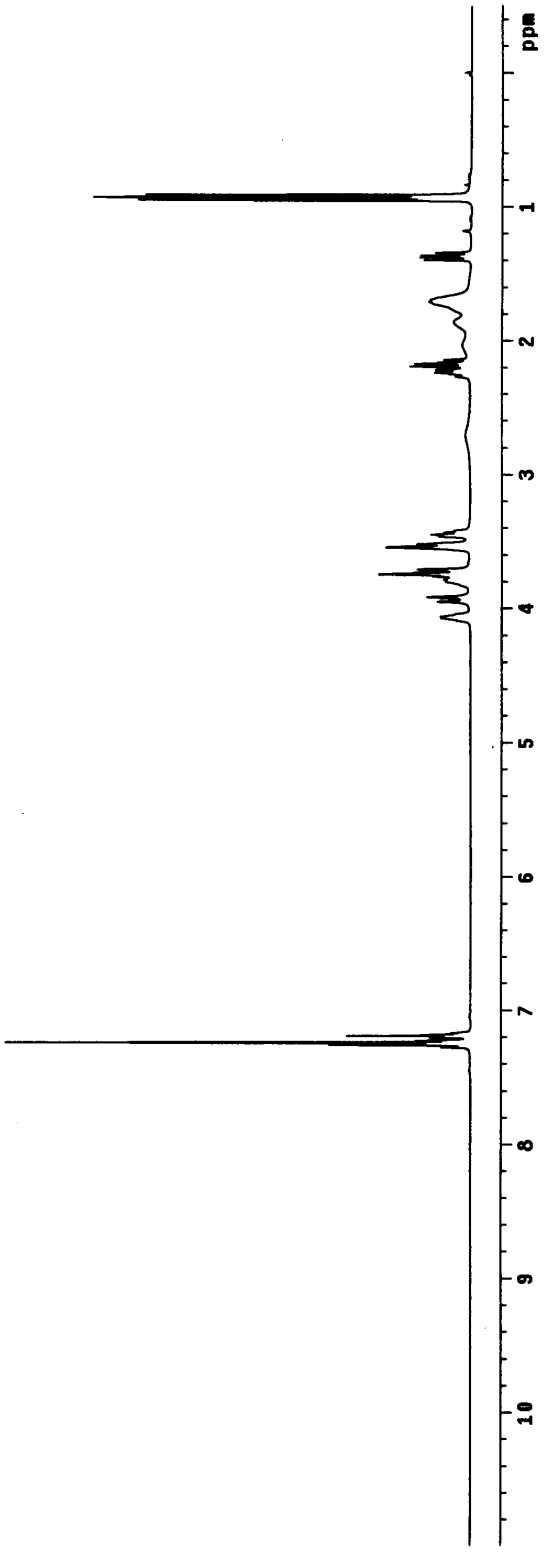

N
O
OTBS
OH
S4
ppm

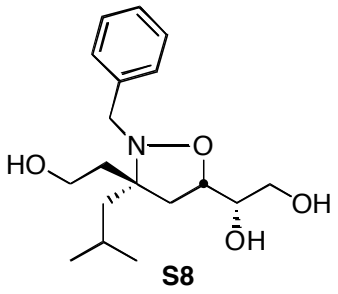

N
O
OTBS
OH
S5
0 ppm
1
2
3
4
5
6
7
8
9
10
8.67
1.03
1.02
1.07
0.98
2.00
2.03
5.20


N
O
OTBS
OH
S6

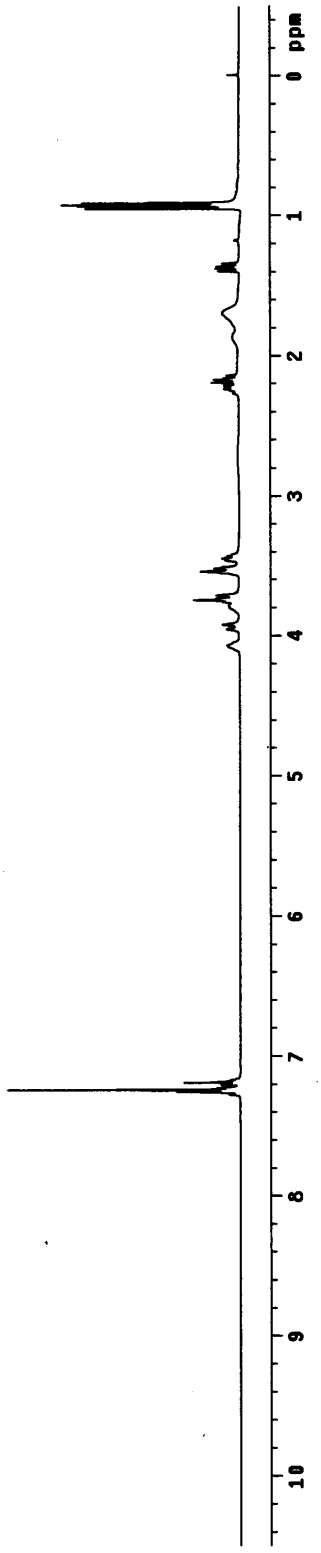


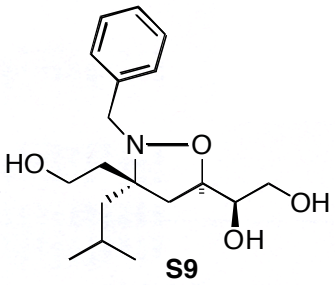
S7



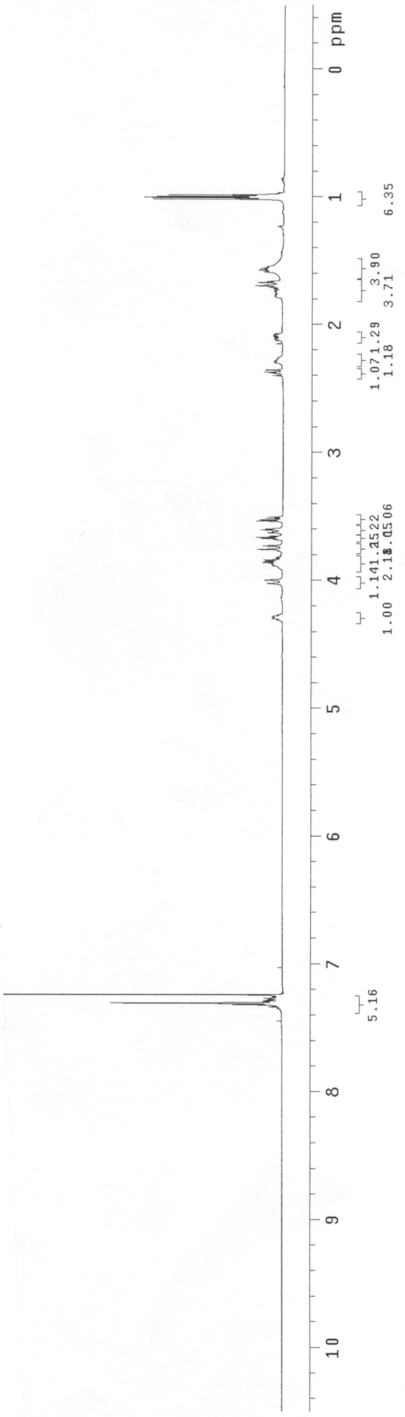


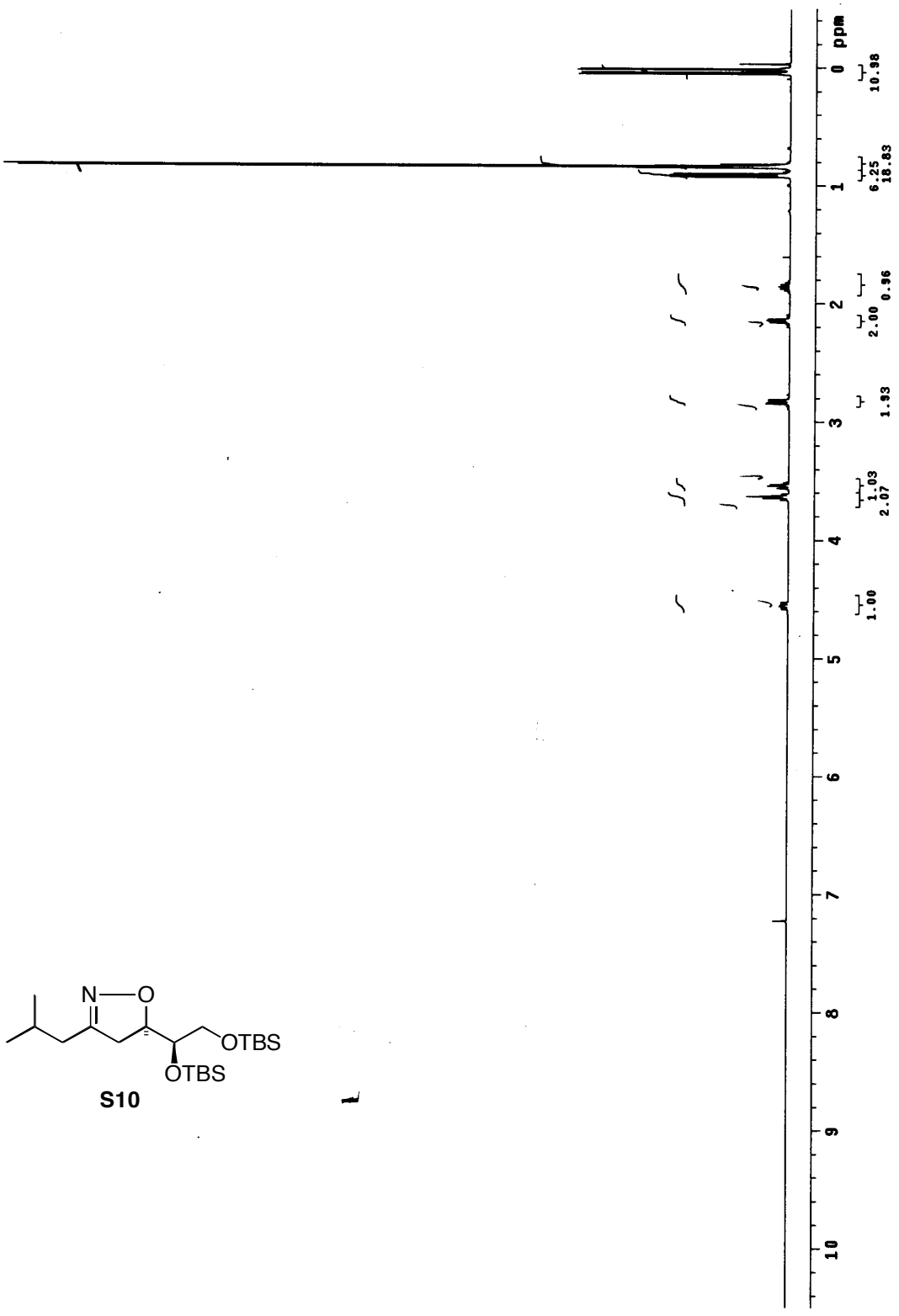
S8

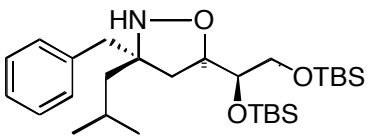



HO
N
O
OH
OH

S9

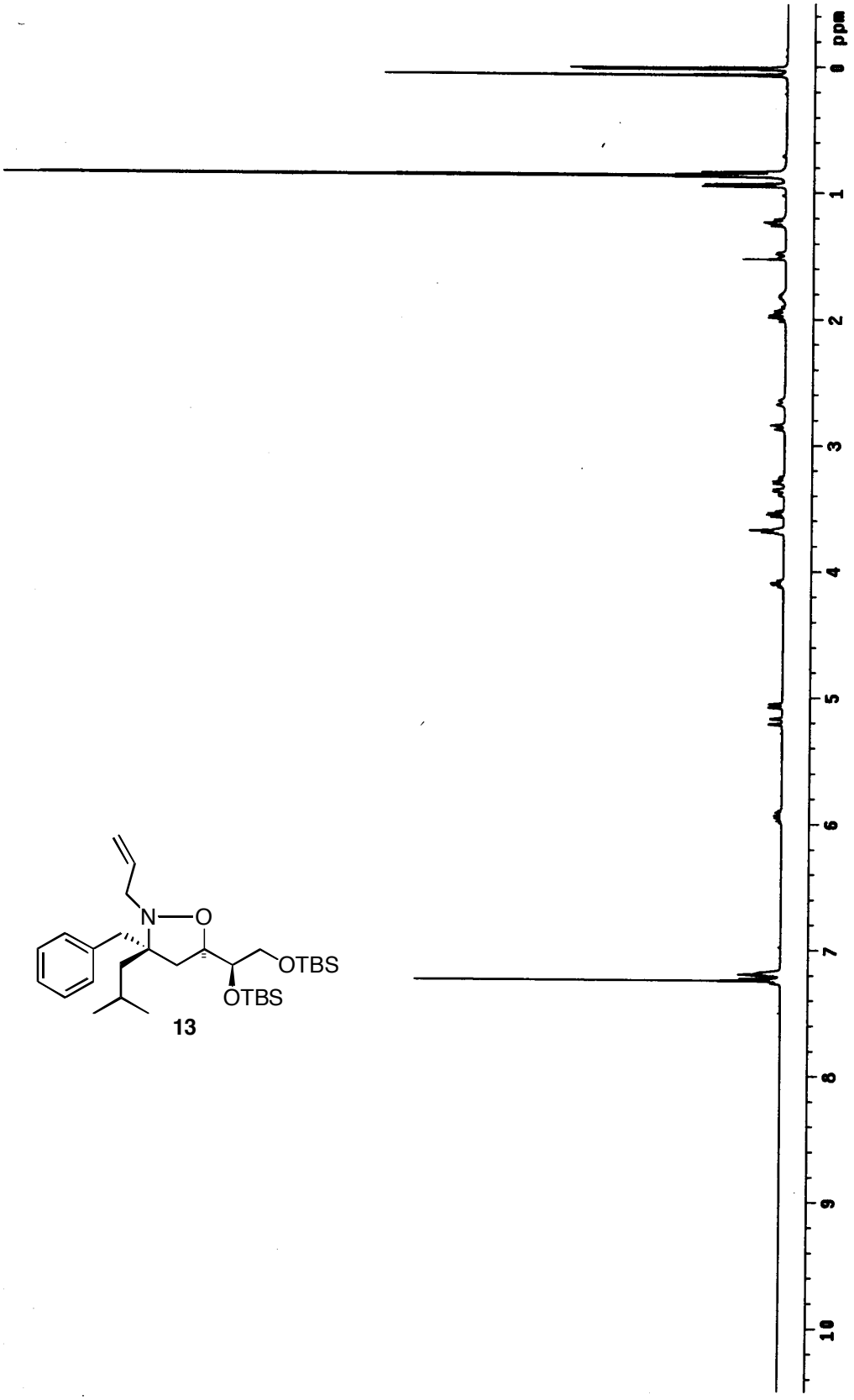

ppm
0
1
2
3
4
5
6
7
8
9
10
5.16
1.00
1.14
1.52
2.18
5.06
1.07
1.29
1.18
3.90
3.71
6.35

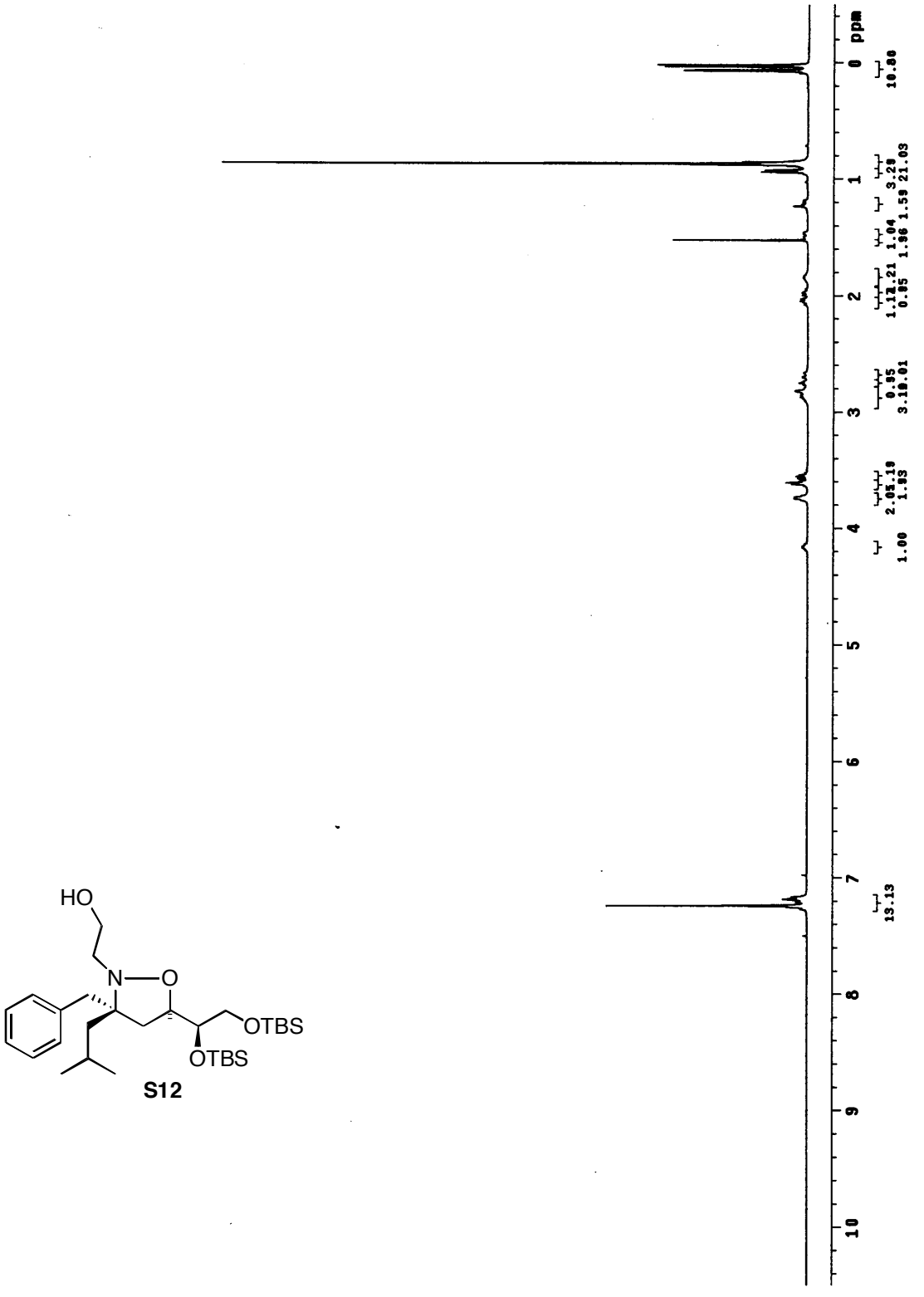

N
O
OTBS
OTBS
S10
0 ppm
10.98
6.25
18.83
2.00
0.96
1.93
1.03
2.07
1.00

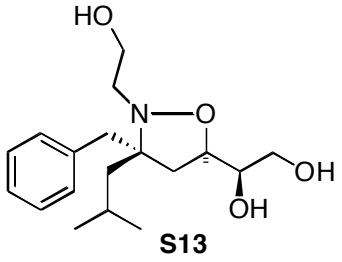

HN
O
OTBS
OTBS

S11

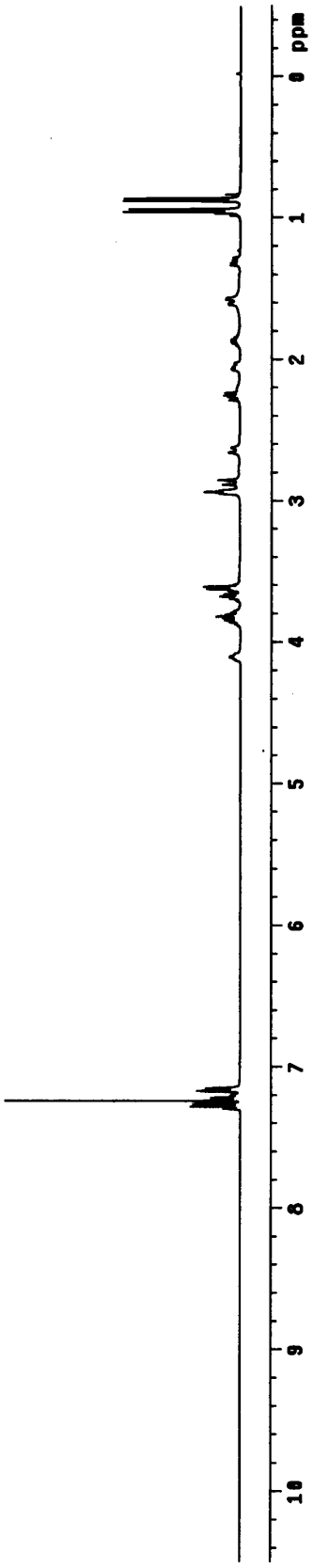
ppm
0
1
2
3
4
5
6
7
8
9
10
11.11
2.68
1.69
0.90
0.91
0.86
0.95
0.95
2.10
0.92
0.81
0.07
1.00
4.16
1.06


N
O
OTBS
OTBS
13
0 ppm
1
2
3
4
5
6
7
8
9
10


HO
N
O
OTBS
OTBS
S12



S13



HPLC traces of compounds **3**-**6**. Compounds **5** and **6** are present as a mixture of E/Z isomers as confirmed by ^{1}H NMR.