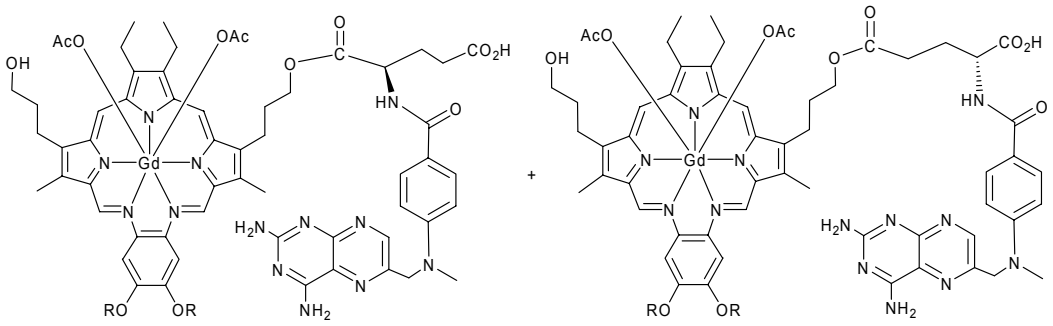
Supplementary Material for Organic & Biomolecular Chemistry

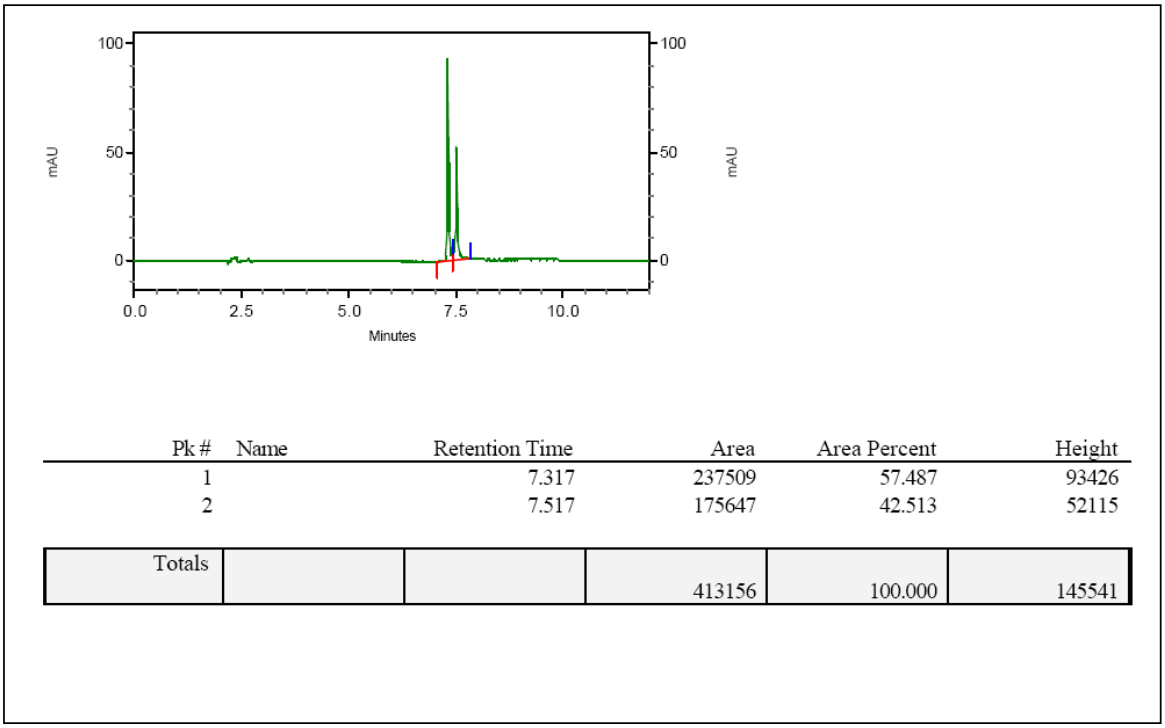
Supplementary data

Supporting Information - Wei, et. al.

MGd-MTX ester conjugate 3 supporting data



The ester conjugate **3**, as previously noted, contains a mixture of both the **3**-α-isomer and the **3**-γ-isomer; the mixture was characterized as noted in the main paper. The



Pk #	Name	Retention Time	Area	Area Percent	Height
1		7.317	237509	57.487	93426
2		7.517	175647	42.513	52115
Totals			413156	100.000	145541

Fig. 1 HPLC trace of α- and γ-esters of MGd-MTX

two products were seen to closely elute on HPLC, (Fig. 1), RT ca. 7.4 min with the difference in retention times varying by 0.2-0.4 min. The two isomers were separated by reverse phase chromatography and the UV spectra overlay of the separated isomers demonstrated identical traces (Fig. 2).

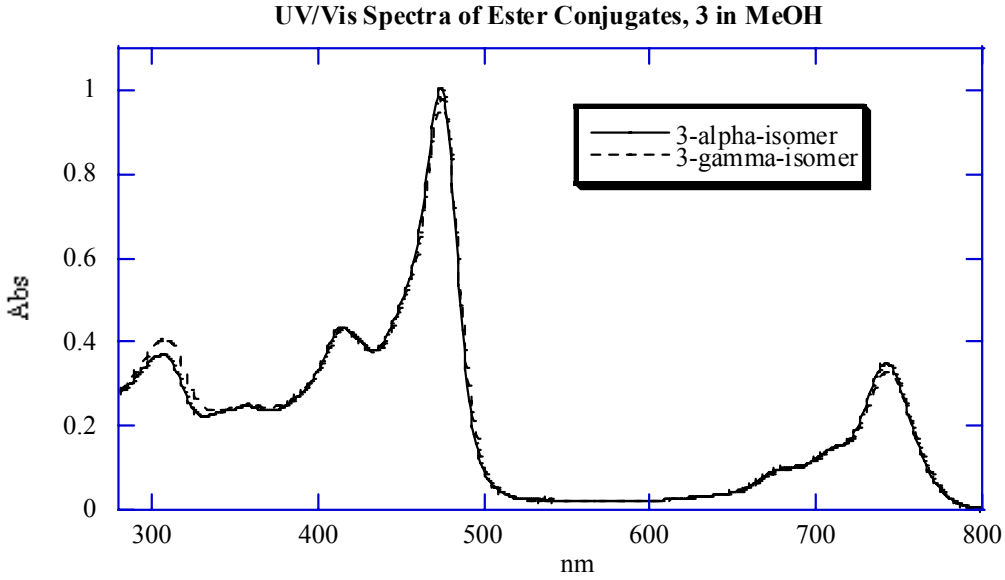


Fig. 2 UV/Vis Spectra of ester conjugates.

HPLC assignment of α- and γ-isomers of MGd-(α,γ)MTX esters

For peak assignments, the MGd-(α)MTX ester **3** was derivatized to the MGd-(α)MTX-(γ)NPr amide (Fig. 3) **S1**, which, following hydrolysis, gave the MTX-(γ)NPr amide **S2**.

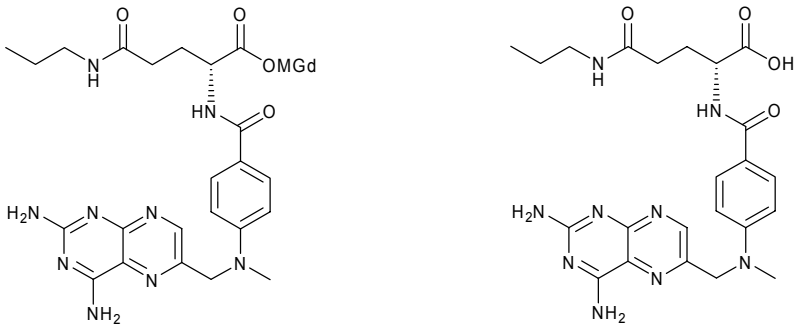
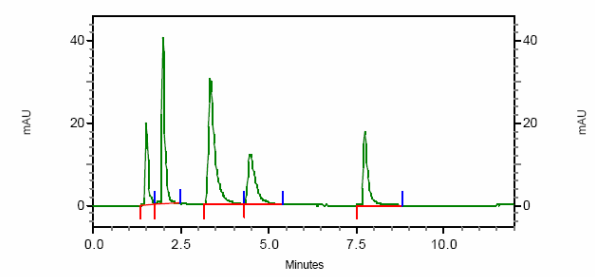


Fig. 3

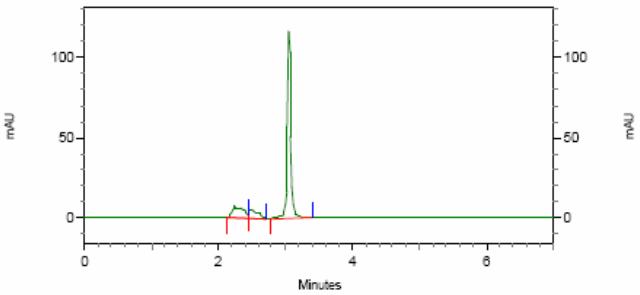
Independent synthesis of the MTX-propylamide as a mixture of the α- and γ-isomers, (Fig. 4) demonstrated HPLC elution in the expected order (impurity, MTX , γ-isomer, α-isomer, bis-α,γ-product). In the case of these derivatives, the γ-isomer and the α-isomer are well separated (3.33 and 4.47 min. respectively). The α- and γ–NPr amides were separated and characterized by MS and NMR (see below).

Confirming the peak assignment, for both the material isolated from the hydrolysis, MTX-(γ)NPr amide derivative **S2** (Fig. 5), and the synthetic γ–isomer **S2**, the HPLC retention times (3.05, 3.08 min. respectively) and MS results proved identical.



Pk #	Name	Retention Time	Area	Area Percent	Height
1		1.500	110989	10.152	19962
2		1.983	247196	22.611	40519
3		3.333	393024	35.950	30678
4		4.467	186626	17.071	12268
5		7.733	155402	14.215	17842
Totals			1093237	100.000	121269

Fig. 4 Crude MTX-NPr synthesis showing (impurity), MTX, γ-isomer, α-isomer, and α,γ-bis-product.



Pk #	Name	Retention Time	Area	Area Percent	Height
1		2.250	87085	15.931	7064
2		2.500	49963	9.140	5588
3		3.050	409575	74.928	116161
Totals			546623	100.000	128813

Fig. 5 MGd-(a)MTX-(g) NPr hydrolysis product, MTX-(g) NPr amide.

S2 Amide Synthesis and Characterization

Synthesis of MTX-propylamide.

To a yellow solution of MTX (20 mg, 0.04 mmol) and propylamine (3.3 □l, 0.04mmol) in dry DMF (1 ml), EDC (9.6 mg, 0.05 mmol) was added at room temperature under argon. The mixture was stirred for 24 hours. The reaction was quenched by adding one drop of AcOH. The mixture was loaded onto tC18 column for first separation. An eluent of MeCN-H_2O was used in a range of 10-50% MeCN, and all the yellow fractions collected and combined. Column chromatography on silica gel with MeOH/DCM yielded three products: MTX-dipropylamide, MTX α-propylamide and MTX γ-propylamide. The yields were not given because the small scale.

By NMR, the amides were largely identical; however, could be differentiated by the shift in the N-18 proton, 8.2 ppm in the case of the α–isomer and 7.6 ppm in the case of the γ–isomer (Fig. 6).

MTX α-propylamide

Tl

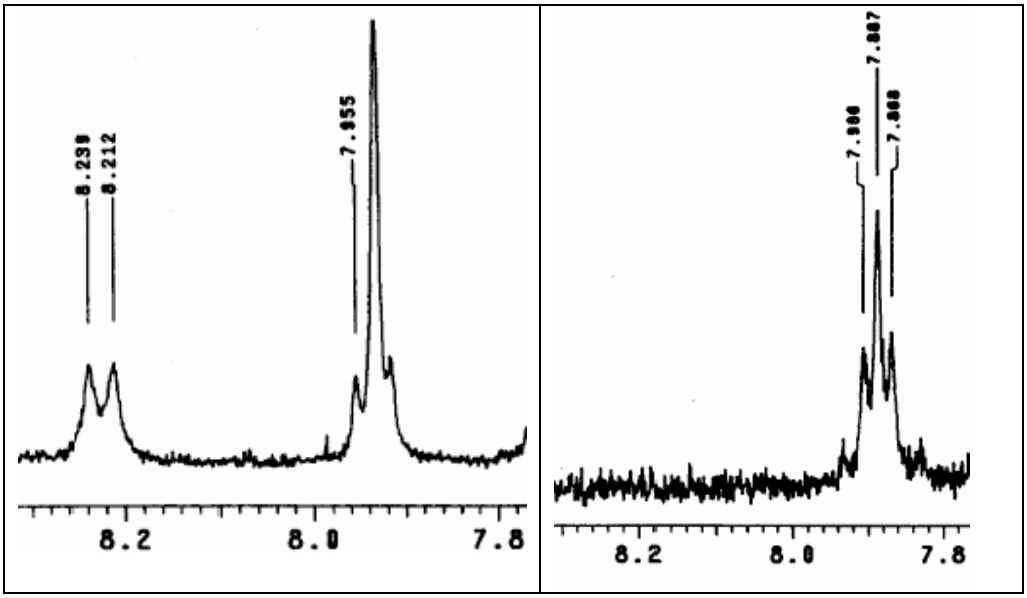


Fig. 6 NMR expansion of the α- and γ-MTX-NPr amides. In the case of the α-amide, the N-18 proton is clearly visible as a doublet at 8.23 ppm.

δ_H (300MHz, DMSO-d_6): 0.79 (t, 3H, *J* = 7.2 Hz), 1.36 (q, 2H, *J* = 7.2 Hz), 1.84-1.96 (m, 2H), 2.20 (t, 2H, *J* = 8.7 Hz), 2.76-3.01 (m, 2H), 3.19 (s, 3H), 4.35-4.28 (m, 2H), 4.77 (s, 2H), 6.78 (d, 2H, *J* = 8.7 Hz), 7.42 (bs, 2H), 7.66 (bs, 2H), 7.74 (d, 2H, *J* = 9.0 Hz), 7.93 (t, 1H, *J* = 5.8 Hz), 8.22 (d, 1H, *J* = 8.1 Hz), 8.54 (s, 1H).

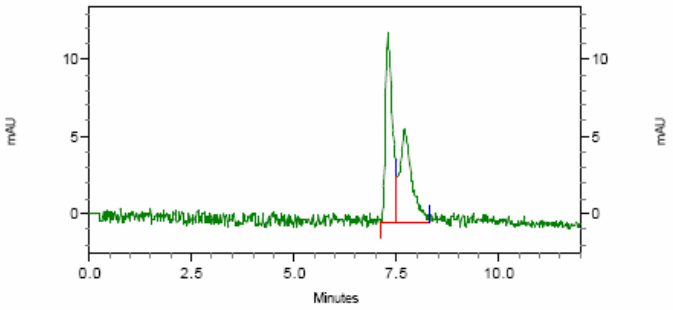
MTX γ-propylamide

δ_H (300MHz, DMSO-d_6): 0.77 (t, 3H, *J* = 7.2 Hz), 1.32 (q, 2H, *J* = 7.2 Hz), 1.83-2.10 (m, 4H), 2.91 (q, 2H, *J* = 6.6 Hz), 3.18 (s, 3H), 3.95-4.10 (m, 2H), 4.75 (s, 2H), 6.59 (bs, 2H), 6.81 (d, 2H, *J* = 8.7 Hz), 7.42 (bs, 2H), 7.59 (overlap, 1H), 7.89 (t, 1H, *J* = 5.8 Hz), 8.54 (s, 1H).

Characterization of MGd-MTX Amide α- and γ-isomers

The amide conjugate **9**-α-isomer and **9**-γ-isomer, like the ester, were closely retained on HPLC (Fig. 7). The separated isomers appeared identical by UV (Fig. 8). Due to lack of biological activity and the hydrolytic stability of the product, no further analyses were required.

HPLC Amide mixture, 9-α- and 9-γ-isomers



Pk #	Name	Retention Time	Area	Area Percent
1		7.300	151725	55.839
2		7.700	119992	44.161
Totals			271717	100.000

Fig. 7 HPLC of amide mixture, 9-α- and 9-γ-isomers.

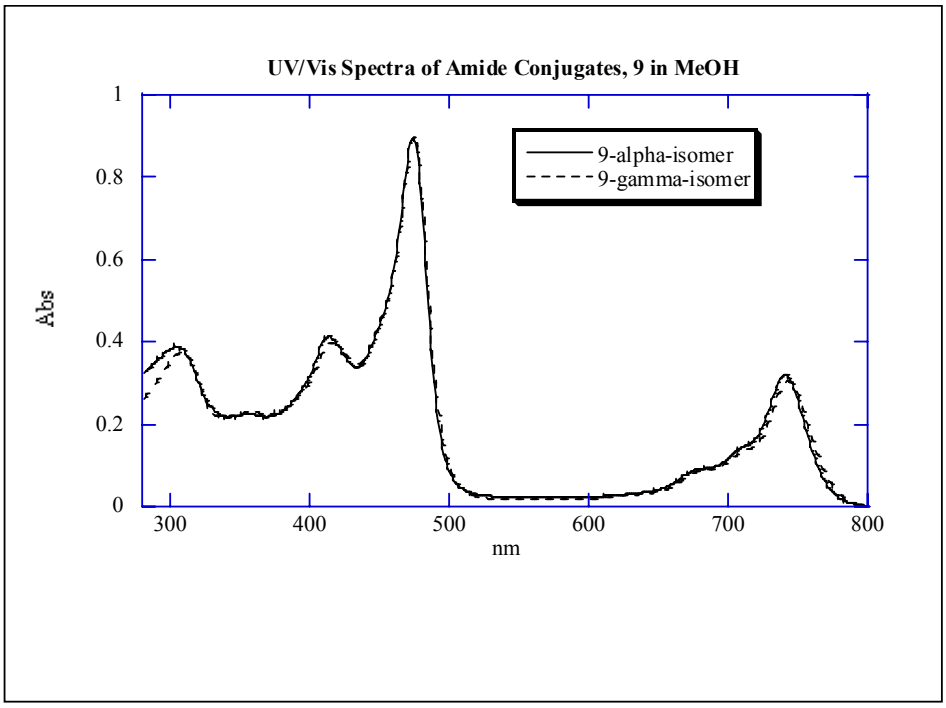


Fig. 8 UV of amide 9-α and 9-γ-isomers

Supporting Information:

To assess recovery, medium (RPMI 1640 supplemented with fetal bovine serum), A549 cell homogenate (5 x 10^6 cells/mL in medium), and water were each fortified with 0.5 or 50 μM of each test article (n=3 replicates per test condition). Each sample was assayed by reversed-phase HPLC. The recovery was determined for each test condition by determining the ratio of test article peak area in matrix (medium or cell homogenate) to water.

To assess conjugate stability, cell pellets and supernatant from plateau phase cultures of A549 cells were sampled after 4 hr, 8 hr, and 24 hr incubation. Samples were stored at –20 ^{o}C for subsequent extraction and analysis using reversed-phase HPLC. Values are the average of 3 measurements.

Both stability and recovery samples were assayed in accordance with standard procedures. The medium and cell pellets were extracted by the addition of a 50/50 v/v solution of methanol/acetonitrile containing 0.16 M glacial acetic acid and zinc sulfate. Extraction efficiency was corrected using an internal standard. HPLC was performed using an Agilent HP1100 chromatography system with detection based on MGd absorbance at 470 nm.

Depending on concentration, texaphyrin species were recovered from cell lysates with an efficiency of 36-60% at 0.5 μM and greater than 90% at 50 μM (see Table 1).

Methotrexate ester and amide species remained substantially (>99%) uncleaved in culture medium over the 24 hour time course of incubation. The amide conjugate associated with the cell pellet remained uncleaved (>99.7%) over this time interval, whereas the corresponding ester conjugate was cleaved to the extent of 9.5 to 17.5%. Although a greater amount of ester conjugate was taken up after 24 hours, the percentage of total conjugate cleaved at this time was lower (9.5%), possibly indicating that the capacity of cell associated esterase activity had been exceeded (see Figure 9).

Table 1.

Test Article	Matrix	Initial Concen-tration (μM)	N	Test Article Mean Peak Area	RSD	% Recovery
Amide Conjugate 9	Cell Homogenate	0.5	3	15.0	1.6 %	59.9 %
Amide Conjugate 9	Complete Medium	0.5	3	25.1	0.4 %	100.1 %
Amide Conjugate 9	Milli-Q Water	0.5	3	25.1	2.6 %	100.0 %
Amide Conjugate 9	Cell Homogenate	50.0	3	2609.7	0.7 %	91.1 %
Amide Conjugate 9	Complete Medium	50.0	3	2845.7	1.1 %	99.4 %
Amide Conjugate 9	Milli-Q Water	50.0	3	2864.0	0.5 %	100.0 %
MGd-Monoamine	Cell Homogenate	0.5	3	8.4	11.8 %	36.1 %
MGd-Monoamine	Complete Medium	0.5	3	23.6	1.7 %	101.1 %
MGd-Monoamine	Milli-Q Water	0.5	3	23.3	0.7 %	100.0 %
MGd-Monoamine	Cell Homogenate	50.0	3	2315.8	1.2 %	91.3 %
MGd-Monoamine	Complete Medium	50.0	3	2551.5	0.4 %	100.6 %
MGd-Monoamine	Milli-Q Water	50.0	3	2535.3	0.8 %	100.0 %
Ester Conjugate 3	Cell Homogenate	0.5	3	15.3	2.1 %	60.7 %
Ester Conjugate 3	Complete Medium	0.5	3	25.3	1.8 %	100.6 %
Ester Conjugate 3	Milli-Q Water	0.5	3	25.1	1.1 %	100.0 %
Ester Conjugate 3	Cell Homogenate	50.0	3	2488.3	1.4 %	90.4 %
Ester Conjugate 3	Complete Medium	50.0	3	2825.1	5.0 %	102.6 %
Ester Conjugate 3	Milli-Q Water	50.0	3	2753.9	0.4 %	100.0 %
MGd	Cell Homogenate	0.5	3	10.0	3.7 %	37.8 %
MGd	Complete Medium	0.5	3	26.4	1.3 %	99.8 %
MGd	Milli-Q Water	0.5	3	26.4	1.8 %	100.0 %
MGd	Cell Homogenate	50.0	3	2471.8	5.8 %	93.6 %
MGd	Complete Medium	50.0	3	2615.7	0.8 %	99.1 %
MGd	Milli-Q Water	50.0	3	2640.0	1.7 %	100.0 %

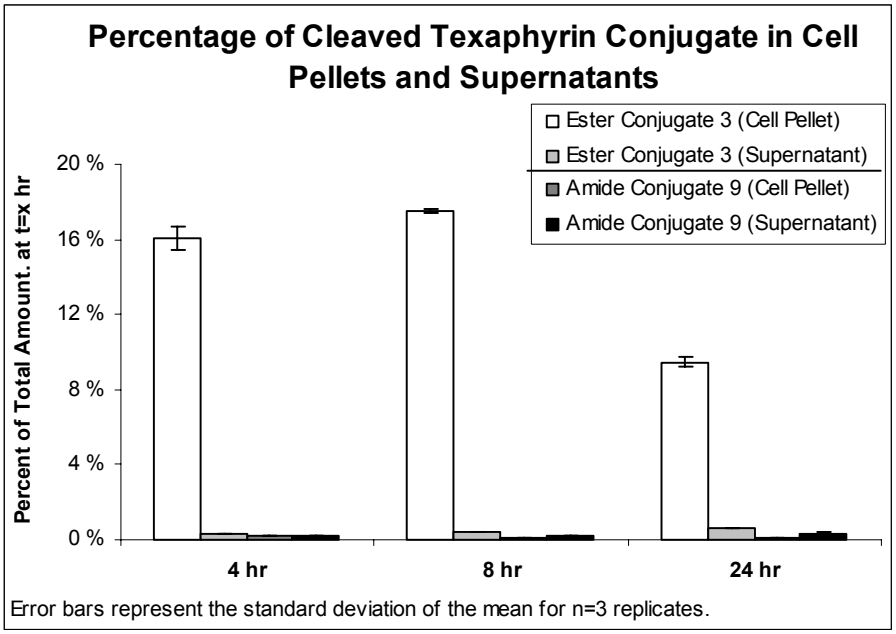


Figure 9.