Supporting Information

Discovery of potent hexapeptide agonists to human neuromedin U receptor 1 and identification of their serum metabolites

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1. Materials

Reagents and solvents were purchased from Wako Pure Chemical Ind. (Osaka, Japan), and Sigma-Aldrich (St. Louis, MO), Nacalai Tesque (Kyoto, Japan), Watanabe Chemical Ind. (Hiroshima, Japan), and Tokyo Chemical Ind. (Tokyo, Japan). All reagents were used as received. Sterile Alfa Modified Eagle Minimum Essential Medium (α-MEM)-Nucleoside, Hank's Balanced Salt Solution (HBSS), and fetal calf serum (FCS) were purchased from Life Technologies (Carlsbad, CA). Sterile 100-mm dishes and 96-well black-walled plates with clear bottoms were purchased from Iwaki (Tokyo, Japan) and Corning (Cambridge, MA), respectively. Rat and human serum were purchased from Sigma-Aldrich. RPMI-1640 was purchased from Wako Pure Chemical Ind. Sep-Pak® C18 Plus cartridges were purchased from Waters (Milford, MA).

2. Synthesis of Peptide Derivatives

The purities of all novel peptides were >95% as assessed by RP-HPLC analysis using a C18 reverse-phase column [4.6 x 150 mm; YMC Pack ODS-AM 302 (column 1), Waters SunFire C18 5 μm (column 2), COSMOSIL 5C_{18}-AR-II (column 3)] with a binary solvent system: a linear gradient of CH_3CN (columns 1 and 2: 5-65%, 40 min; column 3: 10-60%, 100 min) in 0.1% aqueous TFA at a flow rate of 0.9 mL/min (column 1) or 1.0 mL/min (columns 2 and 3), detected by UV at 230 nm (columns 1 and 2) or 220 nm (column 3). Column 3 was used for metabolite analysis. Yields of all products obtained as a white powder were calculated as TFA salts. High-resolution mass spectrometric data (ESI-TOF MS) was obtained using a Micromass LCT. ^{1}H NMR spectra were obtained on a Bruker Avance III spectrometer (400 MHz) with TMS as an internal standard. ^{1}H NMR spectra were obtained on a Bruker Avance III spectrometer (400 MHz) with TMS as an internal standard. Analytical data of novel synthetic peptide derivatives in this study show following;

4b: Yield of 76%; HRMS (TOF MS ES+) *m/z* 973.4824 $(M+H)^+$ (calcd for $C_{46}H_{70}N_{15}O_8$ 973.4830); HPLC (column 2) 100% (t_R = 20.27 min).

4c: Yield of 83%; HRMS (TOF MS ES+) *m/z* 959.4655 $(M+H)^+$ (calcd for $C_{43}H_{65}N_{14}O_8$ 959.4674); HPLC (column 2) 100% (t_R = 18.95 min).

4d: Yield of 69%; ^{1}H NMR (400 MHz, D_2O) δ 7.53 (d, J = 7.9 Hz, 1H), 7.47 (d, J = 8.2 Hz, 1H), 7.33-7.28 (m, 4H), 7.22 (t, J = 7.3 Hz, 1H), 7.12 (t, J = 7.5 Hz, 1H), 7.06-6.92 (m, 4H), 6.77 (d, J = 3.3 Hz, 1H), 4.66 (t, J = 7.6 Hz, 1H), 4.57 (t, J = 6.8 Hz, 1H), 4.50 (t, J = 7.0 Hz, 1H), 4.41 (t, J = 5.5 Hz, 1H), 4.34 (t, J = 6.3 Hz, 1H), 4.25 (t, J = 8.0 Hz, 1H), 3.80-3.49 (m, 4H), 3.21-3.02 (m, 6H), 2.89-2.68 (m, 4H), 2.36-2.24 (m, 1H), 2.09-1.41 (m, 11H); HRMS (TOF MS ES+) *m/z* 998.4792 $(M+H)^+$ (calcd for $C_{44}H_{67}N_{14}O_8$ 998.4783); HPLC (column 2) 100% (t_R = 18.78 min).

4e: Yield of 69%; HRMS (TOF MS ES+) *m/z* 1009.4845 $(M+H)^+$ (calcd for $C_{46}H_{71}N_{14}O_8$ 1009.4830); HPLC (column 2) 100% (t_R = 21.46 min).

4f: Yield of 68%; HRMS (TOF MS ES+) *m/z* 1009.4852 $(M+H)^+$ (calcd for $C_{44}H_{68}N_{15}O_8$ 1009.4830); HPLC (column 2) 100% (t_R = 21.55 min).

5a: Yield of 64%; HRMS (TOF MS ES+) *m/z* 1004.4359 $(M+H)^+$ (calcd for $C_{44}H_{73}N_{14}O_8$ 1004.4347); HPLC (column 2) 97.9% (t_R = 19.02 min).

5b: Yield of 42%; HRMS (TOF MS ES+) *m/z* 988.4701 $(M+H)^+$ (calcd for $C_{45}H_{75}N_{14}O_8$ 988.4688); HPLC (column 2) 99.3% (t_R = 13.07 min).

5c: Yield of 55%; HRMS (TOF MS ES+) *m/z* 999.4706 $(M+H)^+$ (calcd for $C_{46}H_{77}N_{14}O_8$ 999.4736); HPLC (column 2) 100% (t_R = 13.99 min).

5d: Yield of 59%; ^{1}H NMR (400 MHz, D_2O) δ 7.51 (d, J = 8.0 Hz, 1H), 7.46 (d, J = 8.2 Hz, 1H), 7.32 (d, J = 5.2Hz, 1H), 7.21 (t, J = 7.5 Hz, 1H), 7.12 (t, J = 7.7 Hz, 1H), 7.05 (s, 1H), 7.03-6.94 (m, 5H), 6.78 (d, J = 3.3 Hz, 1H), 4.67 (dd, J = 5.6 Hz, 7.8 Hz, 1H), 4.58 (t, J = 6.9 Hz, 1H), 4.46 (t, J = 7.3 Hz, 1H), 4.41-4.30 (m, 2H), 4.25 (dd, J = 5.8 Hz, 8.2 Hz, 1H), 3.80-3.52 (m, 4H), 3.22-3.03 (m, 6H), 2.90-2.69 (m, 4H), 2.39-2.24 (m, 1H), 2.11-1.38 (m, 11H); HRMS (TOF MS ES+) *m/z* 1016.4673 $(M+H)^+$ (calcd for $C_{39}H_{71}N_{12}O_8$ 1016.4689); HPLC (column 2) 100% (t_R = 18.47 min).

1b-m1: Yield of 22%; HRMS (TOF MS ES+) *m/z* 820.4836 $(M+H)^+$ (calcd for $C_{43}H_{73}N_{14}O_8$ 820.4834); HPLC (column 3) 96.7% (t_R = 44.32 min).

4d-m1: Yield of 11%; HRMS (TOF MS ES+) *m/z* 885.4180 $(M+H)^+$ (calcd for $C_{42}H_{63}N_{12}O_8$ 885.4194); HPLC (column 3) 100% (t_R = 41.27 min).

5d-m1: Yield of 39%; HRMS (TOF MS ES+) *m/z* 903.4091 $(M+H)^+$ (calcd for $C_{43}H_{65}N_{12}O_8$ 903.4100); HPLC (column 3) 100% (t_R = 43.09 min).

S1a: Yield of 73%; HRMS (TOF MS ES+) *m/z* 967.5295 $(M+H)^+$ (calcd for $C_{45}H_{69}N_{12}O_8$ 967.5266); HPLC (column 1) 100% (t_R = 18.08 min).

S1b: Yield of 64%; HRMS (TOF MS ES+) *m/z* 953.5118 $(M+H)^+$ (calcd for $C_{43}H_{65}N_{14}O_8$ 953.5110); HPLC (column 1) 100% (t_R = 17.10 min).

S1c: Yield of 95%; HRMS (TOF MS ES+) *m/z* 877.4799 $(M+H)^+$ (calcd for $C_{44}H_{67}N_{14}O_8$ 877.4797); HPLC (column 1) 100% (t_R = 11.59 min).

3. Cell cultures

CHO cells stably expressing NMUR1 or NMUR2 were maintained in α-MEM-nucleotide media with 10% heat-inactivated FCS and 1 mg/mL G418. Subculture was performed every 3-4 days. Cells were grown to approximately 70% confluence on 100-mm dishes and maintained at 37 °C under 5% CO_2.

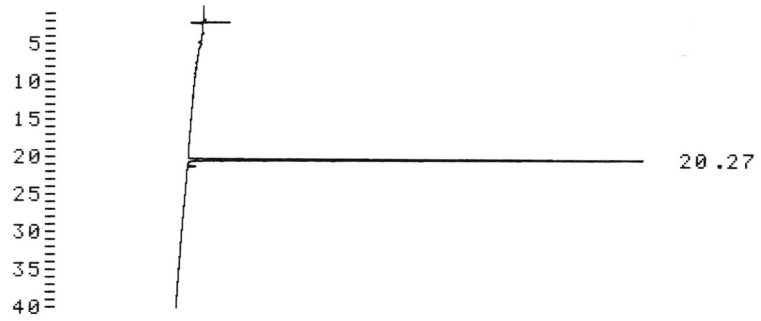
4. Calcium-mobilization assay

CHO cells stably expressing receptors were seeded (2.0 x 10^4 cells per well) into 96-well black-walled plates with clear bottoms. Eighteen hours later, the cells were loaded for 40 min with 4 μM Fluo-4 AM fluorescent indicator dye in assay buffer (HBSS, 10 mM HEPES, 2.5 mM probenecid, pH 7.4) with 1% FCS, and washed four times with the assay buffer without FCS. Then, intracellular calcium flux was assayed on a fluorometric imaging plate reader (Molecular Devices, Sunnyvale, CA). The peptide derivatives were dissolved in an assay buffer containing 0.05% BSA and 0.001% Triton X-100 and prepared at 0.1-1000 nM. The efficacy of the peptide derivatives was determined from the maximal value. To determine EC_{50} values, the peptide derivatives were dissolved at concentrations of 10^{-12}-10^{-6} M. The receptor agonistic activities of the peptide derivatives were determined in triplicate at each concentration.

5. Analysis of metabolic stability in rat/human serum

Each peptide stock solution (20 mM in DMSO) was diluted to 1 mM with RPMI-1640. A 20 μL, 20 nmol aliquot of a 1 mM peptide solution was added to 100 μL rat/human serum diluted with 280 μL RPMI-1640 that had been pre-incubated at 37 °C for 15 min. The resulting 25% rat/human serum solution was incubated at 37 °C, and then the incubation was stopped by the addition of ice-cold saline (400 μL) containing HCl (final conc. 0.04 N). Samples were centrifuged for 3000 rpm at 4 °C for 15 min. Then, an aliquot of supernatant (640 μL) was loaded onto a Sep-Pak® C18 Plus cartridge, and the intact peptide and its metabolites were eluted by 60% CH_3CN in 0.1% aqueous TFA after washing the cartridge with saline and 10% CH_3CN in 0.1% aqueous TFA. Samples were collected into tubes containing 40 μL of a 0.1 % Triton-X 100 solution and lyophilized. Next, samples were dissolved in 800 μL 10% CH_3CN in 0.1% aqueous TFA, and 20 μL was analyzed by RP-HPLC with a C18 reverse-phase column (4.6 x 150 mm; COSMOSIL 5C_{18}-AR-II) with a binary solvent system: a linear gradient of CH_3CN (10-60%, 100 min) in 0.1% aqueous TFA at a flow rate of 1.0 mL/min, detected at UV 220 nm. New peaks were subjected to by high-resolution mass spectrometry to identify the metabolites. Differences in the recovery rates (%) among samples were analyzed using a Student's *t*-test.

Analytical HPLC of derivative **4b**

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D-2500

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  NO.      RT         AREA       CONC  BC
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TOTAL
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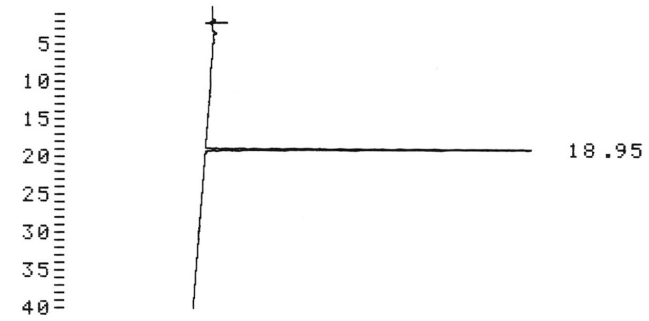
PEAK REJ :        10000
```

Analytical HPLC of derivative **4c**

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D-2500

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TOTAL
                   2669035    100.000
PEAK REJ :        10000
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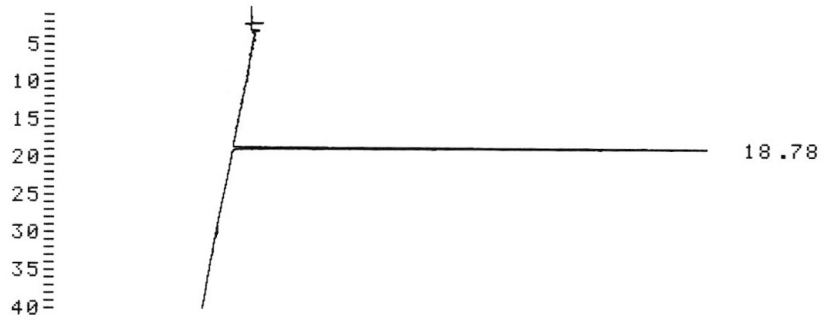
Analytical HPLC of derivative **4d**

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D-2500

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TOTAL
                     1658612    100.000
PEAK REJ :          10000
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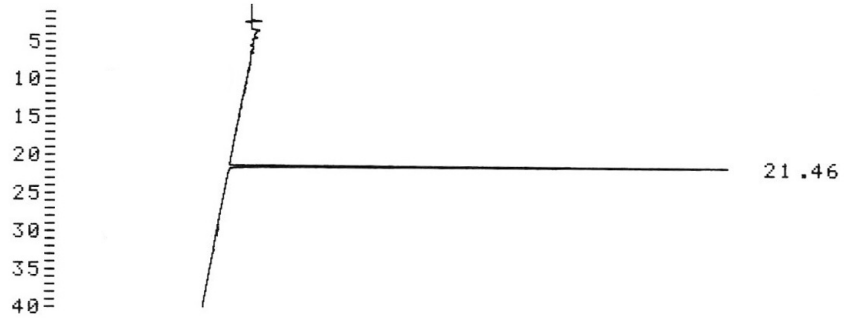
Analytical HPLC of derivative **4e**

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D-2500

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TOTAL
                     1803531    100.000
PEAK REJ :          10000
```

Analytical HPLC of derivative **4f**

D-2500

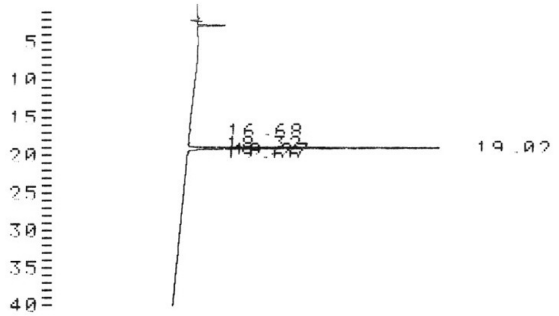
METHOD: TAG: 744 CH: 1

FILE: 0 CALC-METHOD: AREA% TABLE: 0 CONC: AREA

NO.	RT	AREA	CONC	BC
1	21.55	2156106	100.000	BB
TOTAL				
		2156106	100.000	

PEAK REJ : 10000

Analytical HPLC of derivative **5a**



D-2500

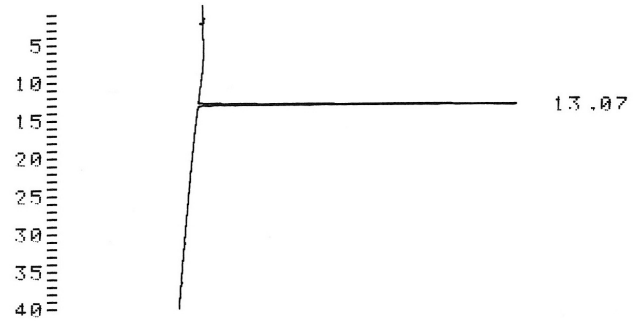
METHOD: TAG: 34 CH: 1

FILE: 0 CALC-METHOD: AREA% TABLE: 0 CONC: AREA

NO.	RT	AREA	CONC	BC
1	16.68	28912	1.184	BB
2	18.32	11227	0.460	BV
3	19.02	2389401	97.882	VV
4	19.27	11566	0.474	TBB
TOTAL				
		2441106	100.000	

PEAK REJ : 10000

Analytical HPLC of derivative **5b**

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D-2500

METHOD:                TAG:    273   CH: 1

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  NO.      RT         AREA       CONC  BC
    1   13.07      2053966     99.299  BB
    3   36.56        14504      0.701  VB
TOTAL
                   2068470    100.000
PEAK REJ :        10000
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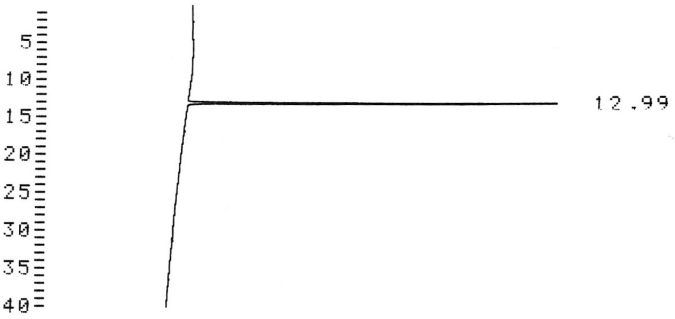
Analytical HPLC of derivative **5c**

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D-2500

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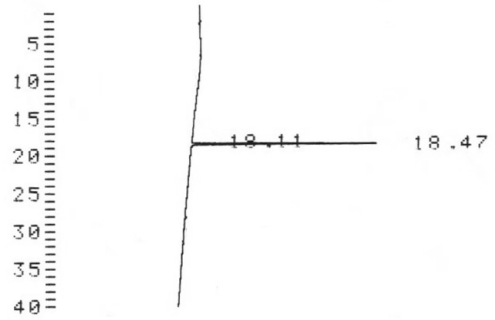
  NO.      RT         AREA       CONC  BC
    1   12.99      2667957    100.000  BB
TOTAL
                   2667957    100.000
PEAK REJ :        10000
```

Analytical HPLC of derivative **5d**



D-2500

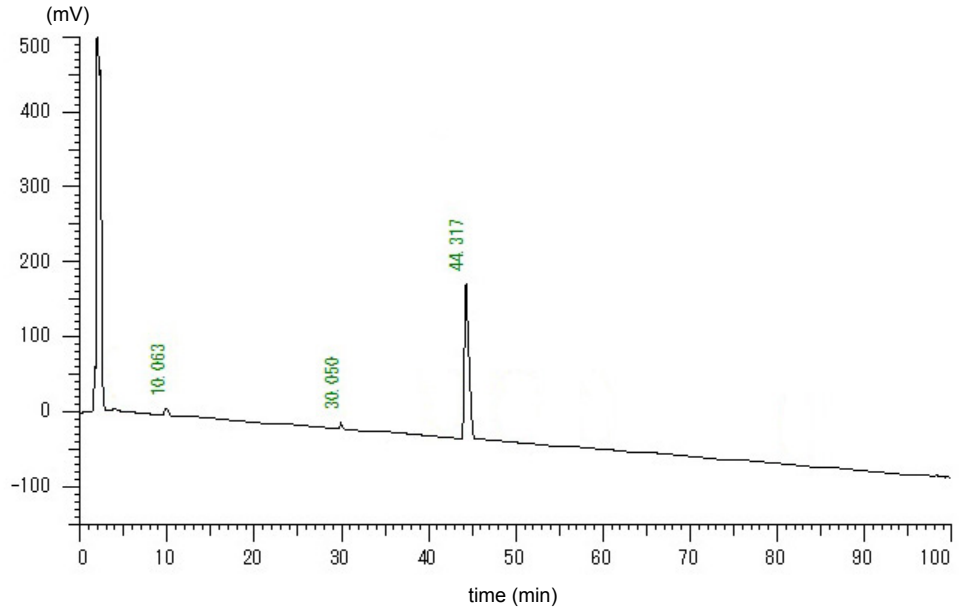
METHOD: TAG: 279 CH: 1

FILE: 0 CALC-METHOD: AREA% TABLE: 0 CONC: AREA

NO.	RT	AREA	CONC	BC
2	18.47	1266615	100.000	BB
TOTAL		1266615	100.000	

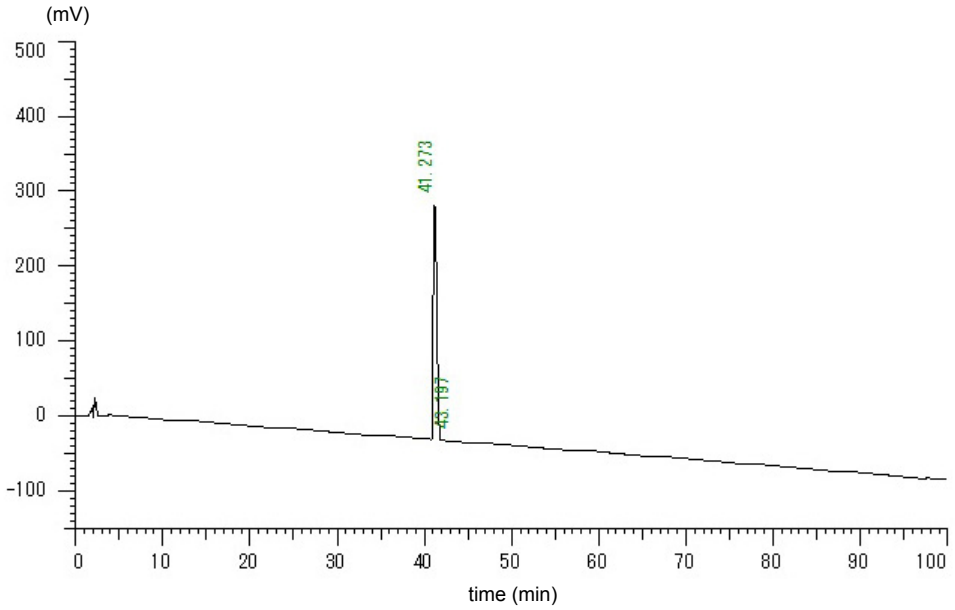
PEAK REJ : 10000

Analytical HPLC of synthetic metabolite **1b-m1**



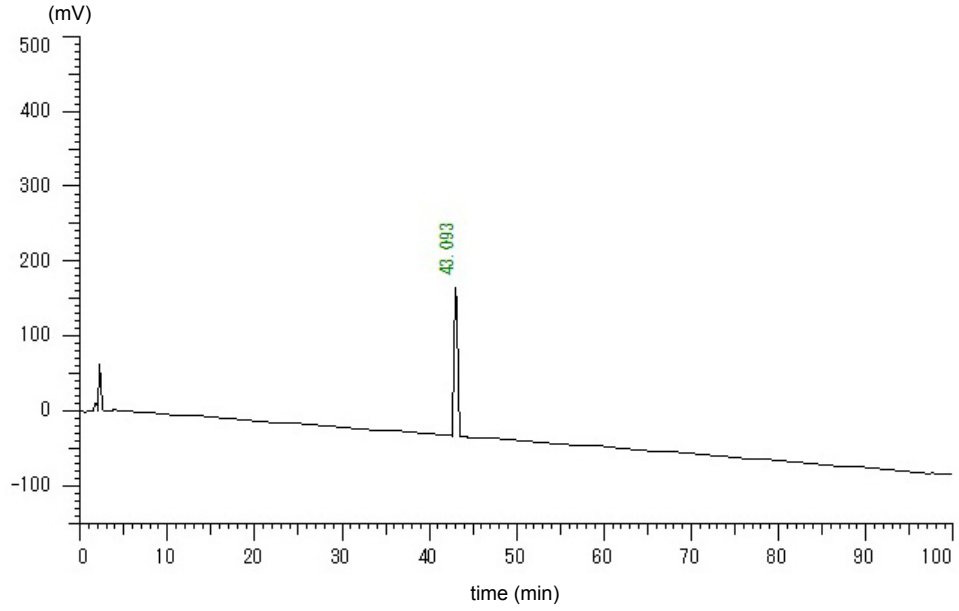
NO	RT	Area	Conc.	BC
1	10.063	106816	1.70	BB
2	30.050	98293	1.57	BB
3	44.317	6061739	96.73	BB
		6266848	100.00	

Analytical HPLC of synthetic metabolite **4d-m1**



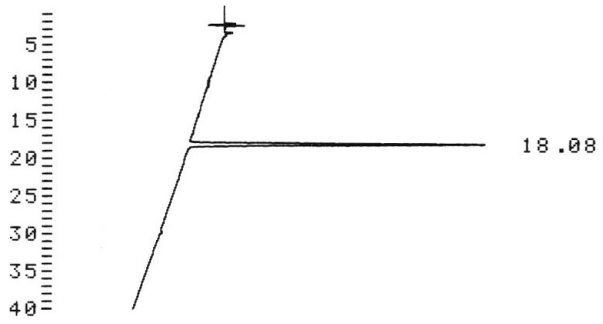
NO	RT	Area	Conc.	BC
1	41.273	5202829	99.988	BB
2	43.197	633	0.012	BB
		5203462	100	

Analytical HPLC of synthetic metabolite **5d-m1**



NO	RT	Area	Conc.	BC
1	43.093	3057648	100	BB
		3057648	100	

Analytical HPLC of derivative **S1a**

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D-2500

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TOTAL
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PEAK REJ :          10000
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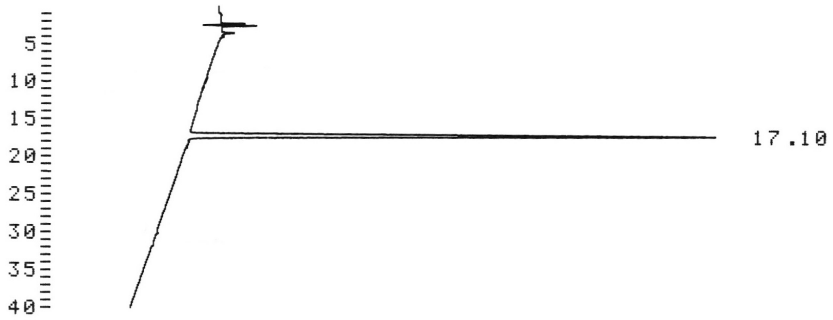
Analytical HPLC of derivative **S1b**

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D-2500

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TOTAL
                     2936452    100.000
PEAK REJ :         100000
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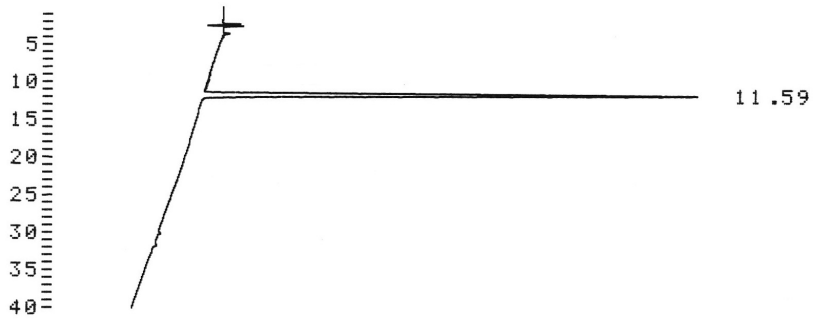
Analytical HPLC of derivative **S1c**



D-2500

METHOD: TAG: 110 CH: 1

FILE: 0 CALC-METHOD: AREA% TABLE: 0 CONC: AREA

NO.	RT	AREA	CONC	BC
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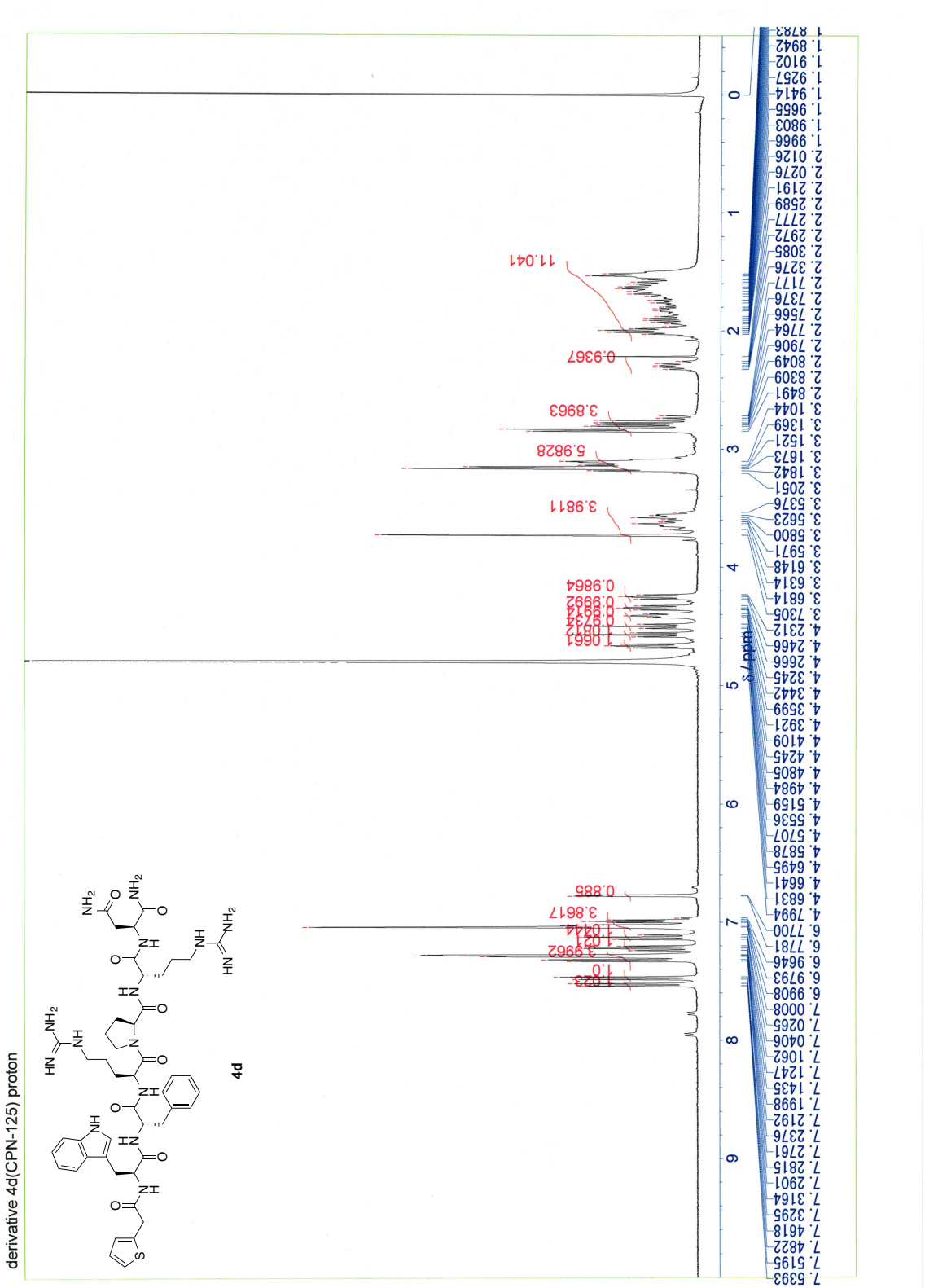
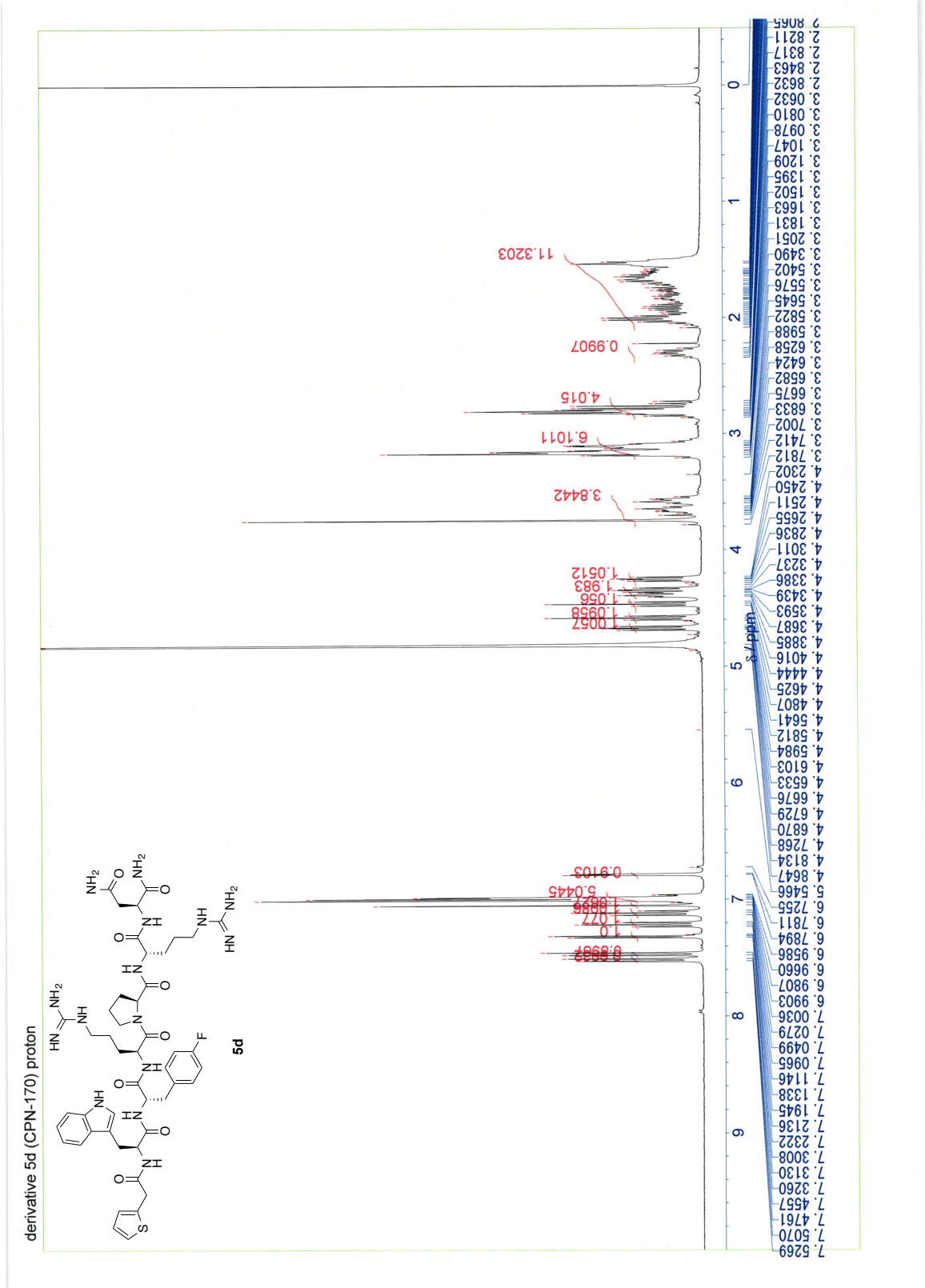
TOTAL				
		2800026	100.000	

PEAK REJ : 100000

^{1}H NMR spectrum of derivative **4d**



[1]H NMR spectrum of derivative **5d**



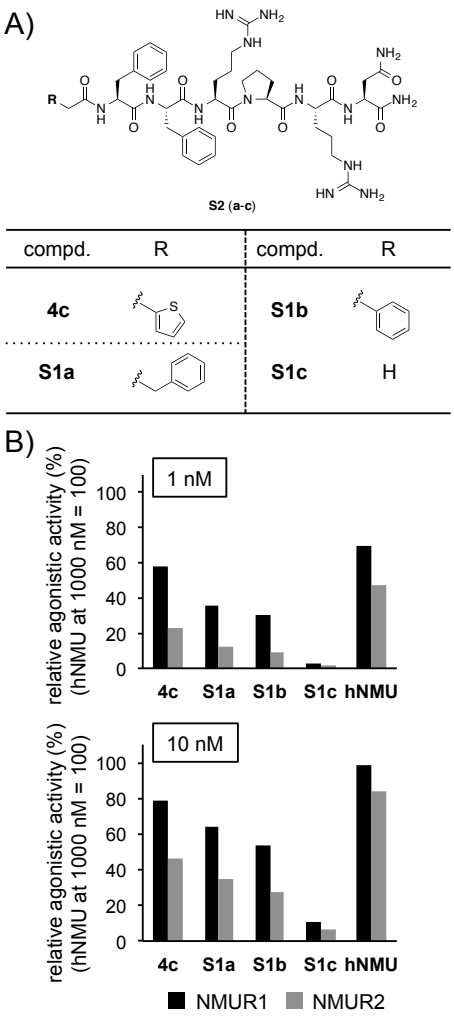


Figure S1. (A) Structures of **S1a**-**S1c**. (B) Effect of substitutions at the *N*-terminus (residue 0) on the agonistic activity in CHO cells toward stably expressed human NMUR1 (*black bar*) and NMUR2 (*gray bar*) as determined by the calcium mobilization assay. Peptide concentration, 1 and 10 nM; positive control, hNMU (activity at 1000 nM = 100%).

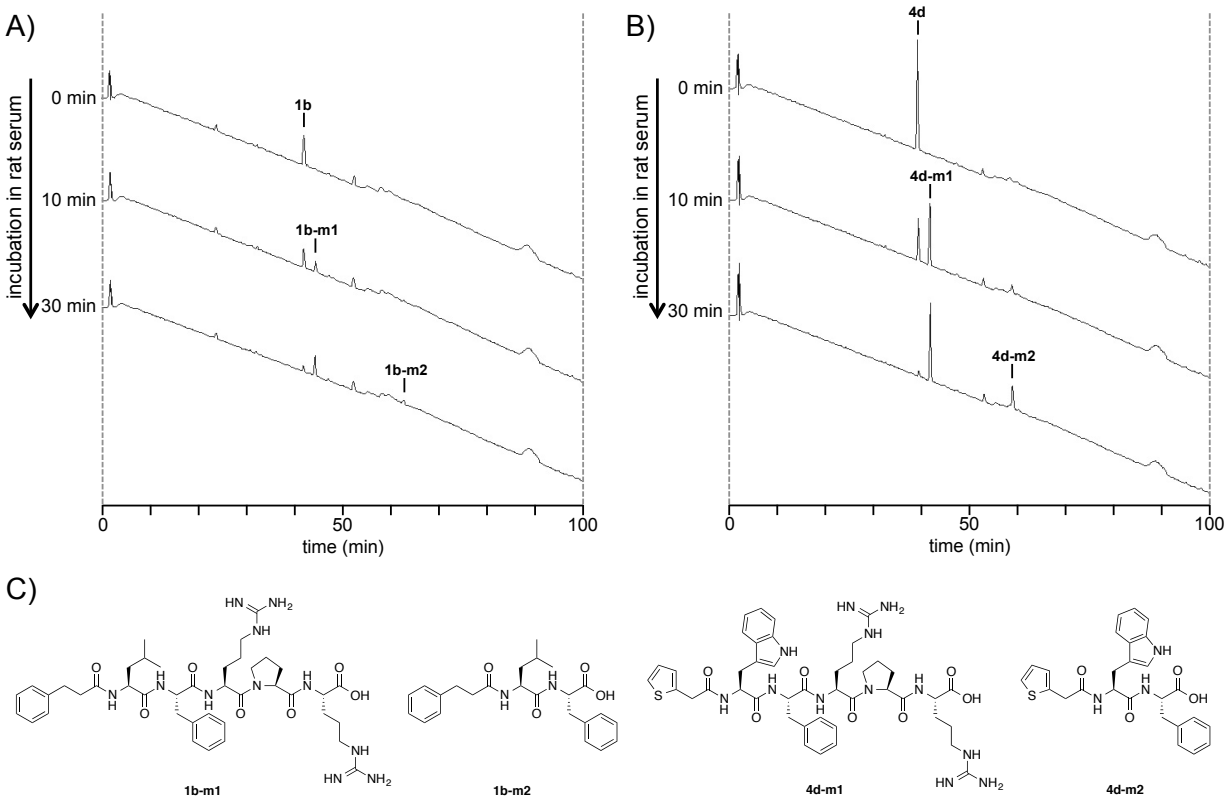


Figure S2. Analytical RP-HPLC chromatograms showing the time-dependent metabolic degradation of **1b** (A) and **4d** (B) in 25% rat serum. An aliquot of each sample extracted by Sep-Pak C18 Plus cartridges was analyzed using a C18 reverse-phase column detected by UV at 220 nm. (C) Structures of **1b**-/**4d**-derived major metabolites **1b-/4d-m1**, and **1b-/4d-m2**.

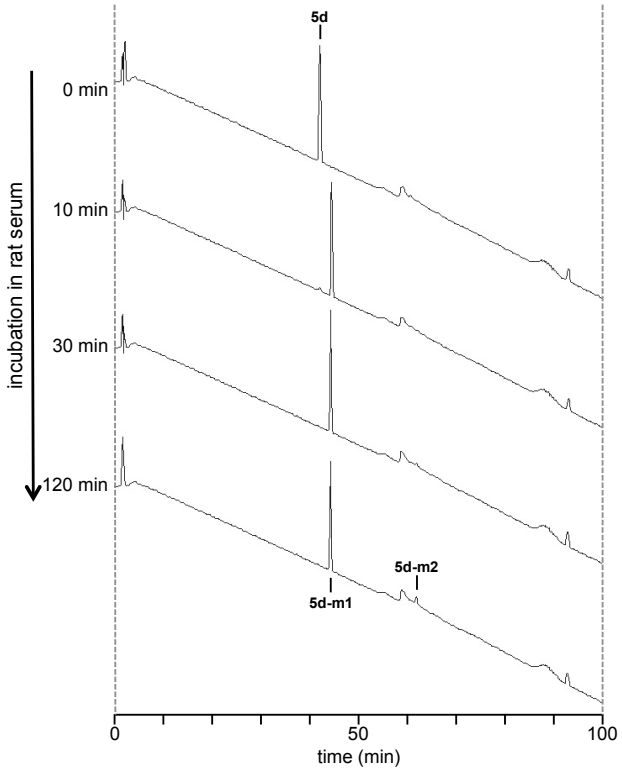


Figure S3. Analytical RP-HPLC chromatograms showing the time-dependent metabolic degradation of **5d** in 25% human serum. An aliquot of each sample extracted by Sep-Pak C18 Plus cartridges was analyzed using a C18 reverse-phase column detected by UV at 220 nm.