Synthesis and *in Vitro* Evaluation of Two Progressive Series of Bifunctional Polyhydroxybenzamide Catechol-*O*-methyltransferase Inhibitors

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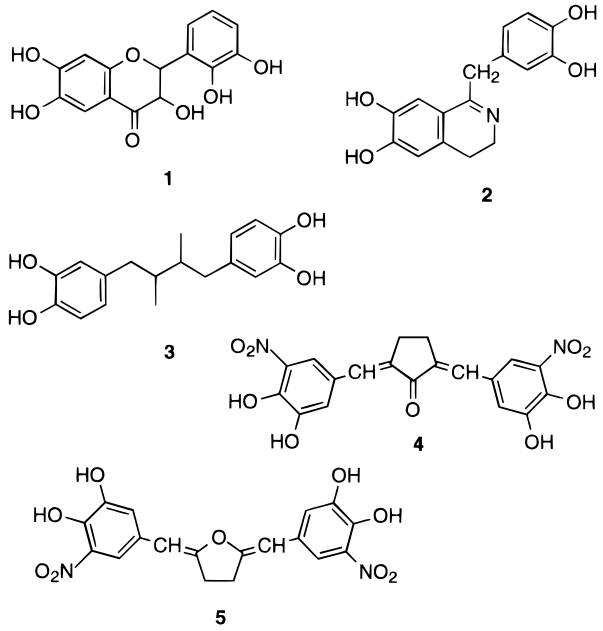
Two progressive series of molecules with two polyhydroxybenzamide substructures were synthesized and tested as potential inhibitors of catechol-*O*-methyltransferase (COMT). These compounds were designed for the purpose of enhanced enzyme binding with duplicated substructures separated by a linker section of various lengths. Our results show that potency and mode of inhibition observed with the "bifunctional" compounds were a reflection of their bifunctional nature. Furthermore, potency and mode of inhibition were dependent on the length of the linker section. Of the assayed compounds, the optimum linker was found to be diaminopropane. For example, *N*,*N*′-1,3-propanediylbis(3,4-dihydroxybenzamide) and *N*,*N*′-1,3-propanediylbis(3,4,5-trihydroxybenzamide) demonstrated strong inhibitory action against COMT, with apparent K_i values of 0.3 and 6.0 μM, respectively.

Introduction

Catechol-*O*-methyltransferase (COMT, EC 2.1.1.6) is a magnesium dependent enzyme which catalyzes the transfer of a methyl group from *S*-adenosylmethionine (AdoMet) to endogenous and exogenous catechols,[1,2] forming *O*-methylated products and *S*-adenosylhomocysteine (SAH).[3] COMT is responsible for the extraneuronal inactivation of catecholamines and the detoxification of many xenobiotic catechols.[4−7] Inhibitors of COMT are of interest for preventing the inactivation of exogenously administered catechols and, therefore, have potential as drugs to combat disorders of catecholamine metabolism such as Parkinson's disease (PD). Orally administered L-dopa (3-(3,4-dihydroxyphenyl)-L-alanine), taken in conjunction with dopa decarboxylase inhibitors[8,9] (DCI's) such as carbidopa and benserazide, is currently used in the treatment of PD. The methylation of L-dopa by COMT results in the formation of the *O*-methylated metabolite 3-*O*-methyldopa (3-OMD). When a DCI is used in conjunction with L-dopa, the level of 3-OMD increases and it becomes the main circulating metabolite in Parkinsonian patients.[10,11] 3-OMD accumulates in the tissues and plasma and competes with L-dopa and other large neutral amino acids for the large neutral amino acid transport system[8,10,13,14] at the blood−brain barrier. Inhibition of COMT would provide a new therapeutic approach for increasing the bioavailability of L-dopa especially when used in conjunction with a DCI.[15]

First generation inhibitors were either competitive substrates for COMT[16−18] or compounds that were isosteric with the catechol ring.[18,19] Some examples were pyrogallol, tropolone, catechin, and gallic acid, all of which had K_i's in the 10^{-5} M range.[2] They generally lacked specificity of action, had low efficacy *in vivo*, and were toxic.[2,20,21] New generation inhibitors, especially nitecapone,[10,22−24] tolcapone,[25] and entacapone,[10,22,26,27] have been shown in clinical tests to be selective and potent COMT inhibitors with IC_{50}'s in the low nanomolar range.[1,28,29] Of the numerous COMT inhibitors that have been reported in the literature, only five are of a "bifunctional" nature, in that they have duplicated substructures for enzyme binding.



D-Catechin (**1**), desmethylpapaverine (**2**) and nordihydroguaiaretic acid (**3**) are first generation inhibitors[7,30] while 2,5-bis(3,4-dihydroxy-5-nitrobenzylidene)cyclopentanone (**4**) and OR-441 (**5**) are new generation inhibitors of high potency.[9,21,31] All five molecules have two substituted catechols for potential enzyme binding, but no correlation has been drawn between their potency and bifunctional nature.

In order to investigate the contribution from a second binding substructure, we synthesized two progressive series of bifunctional compounds and examined their inhibition characteristics. These compounds were designed with dual substituted catechols for enzyme binding separated by a linker section of various lengths. The catechol derivatives were either 3,4-dihydroxybenzamide or 3,4,5-trihydroxybenzamide. These were linked by a spacer section consisting of varying numbers of methylene units. Although it is known that electron-withdrawing substituents (*e.g.* NO_2) potentiate

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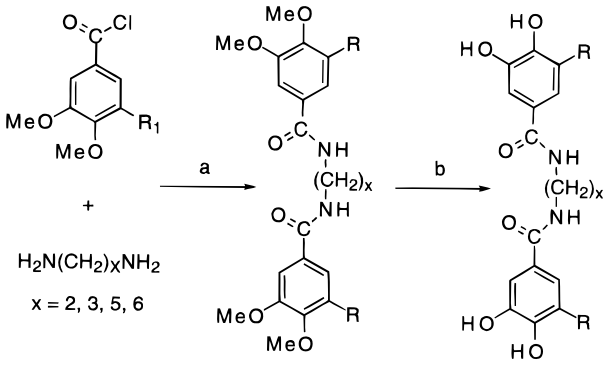
Scheme 1



R	X = 2	3	5	6
H	**6**	**7**	**8**	**9**
OH	**10**	**11**	**12**	**13**

(a) $EtOAc/H_2O/K_2CO_3$; (b) BBr_3/CH_2Cl_2

inhibition,[9,21,31−34] it was decided that the effect of the bifunctional nature of the inhibitors be determined without the addition of enhancement substituents. The results obtained show that maximum potency occurred when the aromatic systems were separated by three methylene spacer groups. Comparison with results obtained with *N*-propyldi- and -trihydroxybenzamides indicates that the bifunctional nature of these compounds greatly affects their potency and mode of action.

Chemistry

The diamide compounds **6**−**13** were prepared by standard procedures: by coupling 3,4-dimethoxybenzoic acid or 3,4,5-trimethoxybenzoic acid with the appropriate diamine followed by demethylation of the polyphenols (Scheme 1). Carboxylic acid to amine coupling was achieved *via* formation of the corresponding benzoyl chloride. Thus, 3,4-dimethoxybenzoic acid and 3,4,5-trimethoxybenzoic acid, both obtained commercially, were reacted with phosphorous pentachloride in dichloromethane to obtain the corresponding benzoyl chlorides. Reaction with an appropriate amount of amine or diamine gave the amides and diamides, respectively. Initial attempts to synthesize the diamide compounds using coupling reagents such as dicyclohexylcarbodiimide (DCC),[35−37] 2-ethoxy-1-(ethoxycarbonyl)-1,2-dihydroquinoline (EEDQ),[38] and ethyl chloroformate under basic conditions,[39] using both methoxy and acetyl protecting groups, were unsuccessful. Coupling reactions with 1,4-diaminobutane using all of the methods described above invariably gave only the monosubstituted product. This result was inexplicable given that in all the other cases diamide products were readily obtained in high yields. Due to this complication, it was decided to proceed without the butyl-linked bifunctional compound in the series. Demethylation to give the hydroxylated derivatives **6**−**13** was achieved through the use of the Lewis acid boron tribromide[40,41] in dichloromethane. An excess was used to allow for complexation of boron tribromide to the ethers and amides. Compounds **6**−**13** were collected from the reaction mixture by filtration and purified by recrystallization from methanol and water. *N*-Propyl-3,4-dihydroxybenzamide (**14**) and *N*-propyl-3,4,5-trihydroxybenzamide (**15**) were synthesized for use as control molecules, using the methodology outlined above with *n*-propylamine in place of diamine.

Biological Results

The compounds synthesized were tested for *in vitro* inhibitory activity with COMT from porcine liver (Sigma, C4512) using methyl 3,4,5-trihydroxybenzoate (methyl gallate) as the methyl group acceptor. The K_m values obtained for AdoMet and methyl gallate were 44.6 and 25.5 μM, respectively. These K_m values are well within the range of reported values for AdoMet and various polyphenol substrates previously described in the literature.[34] Table 1 shows the structure of the bifunctional compounds tested and their inhibition constants with respect to methyl gallate.

Within the 3,4-dihydroxybenzamide bifunctional series (compounds **6**−**9**), the molecule with a spacer section of three methylene units (**7**) was the only inhibitor. It exhibited a competitive inhibition pattern with a K_i of 0.3 μM, which is comparable to many nitrocatechol inhibitors. This result shows that, in this particular series, the length of linker between the aromatic substructures is critical for interaction with COMT. In comparison, compound **14**, with only one 3,4-dihydroxybenzamide moiety and a propyl substituent on the amide nitrogen, was not an inhibitor of COMT at concentrations up to 43.5 μM. The lack of activity displayed by **14** toward COMT, with respect to methyl 3,4,5-trihydroxybenzoate, indicates that the observed inhibition by compound **7** is due to its bifunctional nature.

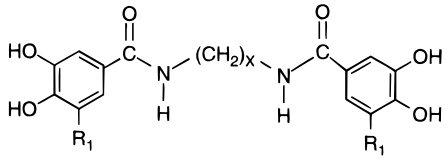
All of the compounds tested in the 3,4,5-trihydroxybenzamide bifunctional series (compounds **10**−**13**) showed an uncompetitive inhibition pattern with respect to methyl gallate. The most potent inhibitor in this series was compound **11** with a spacer section of three methylene units. The K_i measured was 6.6 μM. By comparison, the *N*-propyl analogue **15** demonstrated a purely competitive mode of inhibition with a K_i value of 0.5 μM. Although **15** is a more potent inhibitor than its bifunctional analogue **11** it has a distinctly different mode of action.

Within the two families of bifunctional compounds studied, the linker which confers the greatest inhibitory activity is *n*-propyl. However, the two most potent inhibitors from each group (compounds **7** and **11**) exhibit different modes of inhibition, presumably due to distinct interactions from 3,4-dihydroxy- and 3,4,5-trihydroxybenzamides. Interestingly, the control molecules **14** and **15** interact differently with the active site of COMT than their bifunctional analogues. Presumably, the second tethered binding substructure alters the interaction of the molecule with the enzyme. These results show that duplication of a binding substructure within one molecule can have a profound effect on interaction with the active site of COMT. Furthermore, the effect is greatly affected by the nature of the linking structure.

Experimental Section

COMT Assay. The activity of COMT was measured by determining the amount of methyl 3,5-dihydroxy-4-methoxybenzoate formed over time using HPLC with ultraviolet detection. The standard incubation mixture was 60 μL containing 2 mM AdoMet, 0.02−0.27 mM methyl 3,4,5-trihydroxybenzoate, COMT (100 units, where one unit is defined as the methylation of 1 nmol of protocatechuic acid

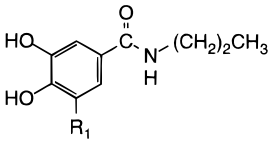
Table 1. Structure, COMT Inhibitory Pattern, and Kinetic Inhibition Constants of the Compounds Synthesized



compd	R_1	*x*	% yield	mp, °C	formula	anal.	inhibition pattern	kinetic inhibition constant, K_i/μM
6	H	2	27	236[a]	$C_{16}H_{16}N_2O_6$	C,H,N	NA[b]	
7	H	3	45	244	$C_{17}H_{18}N_2O_6$	C,H,N	C[c]	0.30 ± 0.013
8	H	5	57	210	$C_{19}H_{22}N_2O_6 \cdot {}^1/_2H_2O$	C,H,N	NA	
9	H	6	34	234[a]	$C_{20}H_{24}N_2O_6$	C,H,N	NA	
10	OH	2	16	230[a]	$C_{16}H_{16}N_2O_8 \cdot 2{}^1/_4H_2O$	C,H,N	UC[d]	136 ± 10.5
11	OH	3	26	210[a]	$C_{17}H_{18}N_2O_8 \cdot 4H_2O$	C,H,N	UC	6.6 ± 0.32
12	OH	5	58	210[a]	$C_{19}H_{22}N_2O_8 \cdot 2H_2O$	C,H,N	UC	26.7 ± 1.6
13	OH	6	28	210[a]	$C_{20}H_{24}N_2O_8 \cdot 2{}^1/_2H_2O$	C,H,N	UC	35.0 ± 3.2

[a] Decomposes. [b] No inhibitory action. [c] Competitive mode of inhibition. [d] Uncompetitive mode of inhibition.

Table 2. Structure, Inhibition Pattern, and Kinetic Inhibition Constants of the *N*-Propyl Derivatives



compd	R_1	inhibition pattern	kinetic inhibition constant, K_i/μM
14	H	NA[a]	
15	OH	C[b]	0.5 ± 0.022

[a] No inhibitory action. [b] Competitive mode of inhibition.

per hour at 37 °C using AdoMet as the methyl donor (Sigma)), buffer[23] (0.2 M NaH_2PO_4, 5 mM $MgCl_2 \cdot 6H_2O$, pH adjusted to 7.45 with NaOH). The mixture was incubated for 1 h at 37 °C, stopped by the addition of 20 μL of 4 M perchloric acid, and then diluted with 200 μL of buffer. A 30 μL aliquot of the assay mixture was injected onto a Microsorb-MV column (C_{18}, 5 μm particle size, 4.6 × 250 mm), and the components of the mixture were eluted with a mobile phase which consisted of buffer (50 mM NaH_2PO_4, 20 mM citric acid)−MeOH−THF (36:20:1 v/v/v)[23] at pH = 2.0, at a flow rate of 0.8 mL/min. Detection was at 270 nm, and the product had a retention time of 21 min. The enzyme activity was calculated as nmol of methyl 3,5-dihydroxy-4-methoxybenzoate/min/μg of protein. Assays were performed in duplicate, and each assay had two 30 μL aliquots analyzed. Conversion of methyl 3,4,5-trihydroxybenzoate to methyl 3,5-dihydroxy-4-methoxybenzoate was less than 5%. The concentrations of AdoMet and Mg^{2+} used were saturating, and product formation was linear with respect to protein concentration and reaction time. Bifunctional inhibitors were tested at concentrations up to 33.9 μM. Compounds which exhibited inhibitory activity less than the standard deviation at this concentration were classified as having no inhibitory action (Table 1). The highest concentrations of compounds **14** and **15** tested were 43.5 and 47.3 μM, respectively.

Analysis of Kinetic Data. All kinetic data were analyzed graphically using double reciprocal plots, where the reciprocal velocities versus the reciprocal substrate concentrations gave a linear relationship by which K_m and V_{max} could be calculated. Inhibition constants for competitive and uncompetitive inhibition were calculated from data fitting to equations $v = V_{max}[S]/(K_m(1 + [I]/K_i) + [S])$ and $v = V_{max}[S]/(K_m + [S](1 + [I]/K_i))$, respectively. Standard deviations were obtained from fitting of all available data for a given compound.

Chemical Methods. Melting points were recorded on a Gallenkamp capillary melting point apparatus and are uncorrected. Elemental analyses were performed at the Campbell Microanalytical laboratory, Otago University. Infrared spectra were recorded on a Biorad FTS-7 spectrometer using KBr disks. ^{1}H NMR and ^{13}C NMR spectra were measured on a Varian Gemini-200 MHz spectrometer. The δ values are reported in ppm downfield from TMS. Column chromatograpy was performed with silica gel (Sorbsil, particle size 32−63 μM) and Dowex ion-exchange resin (counterion H, 100−200 mesh). AdoMet as the *p*-toluenesulfonate salt, COMT, and DL-dithiothreitol (DL-DTT) were purchased from Sigma. 3,4-Dimethoxybenzoic acid, 3,4,5-trimethoxybenzoic acid, methyl 3,4,5-trihydroxybenzoate (methyl gallate), boron tribromide, 1,2-diaminoethane, 1,3-diaminopropane, 1,4-diaminobutane, 1,5-diaminopentane, and 1,6-diaminohexane were purchased from Aldrich. Solvent and inorganic bases were generally sourced.

General Procedure for the Preparation of *N,N′*-Bis(3,4-dihydroxybenzamide) and *N,N′*-Bis(3,4,5-trihydroxybenzamide) Derivatives. 3,4-Dimethoxybenzoic acid or 3,4,5-trimethoxybenzoic acid (recrystallized, 1.0−5.0 g scale) and phosphorus pentachloride (1 molar equiv) were stirred in dichloromethane (100 mL) overnight. The reaction mixture was washed with a saturated solution of sodium hydrogen carbonate, and the organic phase was dried and evaporated under reduced pressure to afford the acid chloride. Freshly prepared acid chloride (2.0−6.0 g scale) and potassium carbonate (1.2 molar equiv) were dissolved in a mixture of water and ethyl acetate (1:1, 70 mL total) and treated with the appropriate diamine (0.5 molar equiv). The resulting biphasic solution was stirred overnight. The solid *N,N′*-bis(3,4-dimethoxybenzamide) product was collected by filtration and dried. Boron tribromide (10 molar equiv) was added to a cooled (0−5 °C) and stirring solution of the appropriate *N,N′*-bis(3,4-dimethoxybenzamide) derivative (0.4−1.0 g scale) in CH_2Cl_2 under anhydrous conditions. Stirring overnight at room temperature resulted in the separation of a white solid. Filtration and washing with copious quantities of CH_2Cl_2 afforded the derivative as a white solid.

***N,N′*-1,2-Ethanediylbis(3,4-dihydroxybenzamide) (6).** The ethylene derivative **6** was prepared from 3,4-dimethoxybenzoic acid (2.0 g) and ethylenediamine using the procedure described above. Recrystallization from MeOH/H_2O gave the desired product as fine white crystals (0.49 g, 27%): mp 235−236 °C dec; IR 3500, 3438, 3376, 3145, 1573, 1559, 1521, 1391, 1327, 1194, 1124 cm^{-1}; ^{1}H NMR (CD_3OD) δ 3.55 (4H, bs), 6.80 (2H, d, J = 8.3 Hz), 7.23 (2H, dd, J = 2.1, 8.3 Hz), 7.29 (2H, d, J = 2.1 Hz); ^{13}C NMR (CD_3OD) δ 41.00, 115.75, 115.88, 120.64, 126.92, 146.28, 150.18, 170.79.

***N,N′*-1,3-Propanediylbis(3,4-dihydroxybenzamide) (7).** The propane derivative **7** was prepared from 3,4-dimethoxybenzoic acid (2.0 g) and 1,3-diaminopropane using the procedure described above. Recrystallization from MeOH/H_2O gave the desired product as a white solid (0.86 g, 45%): mp 243−244 °C; IR 3373, 3198, 1623, 1590, 1546, 1519, 1435, 1311, 1119 cm^{-1}; ^{1}H NMR (CD_3OD) δ 1.84 (2H, m), 3.43 (4H, bm), 6.80 (2H, d, J = 8.2 Hz), 7.22 (2H, dd, J = 2.2, 8.2 Hz), 7.31

(2H, d, $J = 2.2$ Hz); ^{13}C NMR (CD_3OD) δ 30.56, 38.17, 115.73, 116.00, 120.68, 127.02, 146.25, 150.11 170.48.

***N,N′*-1,5-Pentanediylbis(3,4-dihydroxybenzamide) Hydrate (8).** The pentane derivative **8** was prepared from 3,4-dimethoxybenzoic acid (5.0 g) and 1,5-diaminopentane using the procedure described above. Recrystallization from MeOH/H_2O gave the desired product as a white powder (2.8 g, 57%): mp 210−211 °C; IR 3532, 3376, 3118, 1575, 1515, 1455, 1319, 1117 cm^{-1}; ^{1}H NMR (CD_3OD) δ 1.44 (2H, bm), 1.64 (4H, bm), 3.35 (4H, m), 6.78 (2H, d, $J = 8.2$ Hz), 7.19 (2H, dd, $J = 2.2$, 8.2 Hz), 7.28 (2H, $J = 2.2$ Hz); ^{13}C NMR (CD_3OD) δ 25.38, 30.26, 40.77, 115.50, 115.91, 120.47, 126.58, 126.77, 146.58, 146.68, 150.71, 170.40.

***N,N′*-1,6-Hexanediylbis(3,4-dihydroxybenzamide) (9).** The hexane derivative **9** was prepared from 3,4-dimethoxybenzoic acid (4.0 g) and 1,6-diaminohexane using the procedure described above. Recrystallization from MeOH/H_2O gave the desired product as a white solid (1.4 g, 34%): mp 234−235 °C dec; IR 3499, 3370, 3196, 1613, 1575, 1542, 1504, 1431, 1164, 1106 cm^{-1}; ^{1}H NMR (CD_3OD) δ 1.43 (4H, bm), 1.62 (4H, bm), 3.35 (4H, bt), 6.79 (2H, d, $J = 8.2$ Hz), 7.19 (2H, dd, $J = 2.1$, 8.2 Hz), 7.27 (2H, d, $J = 2.1$ Hz); ^{13}C NMR (CD_3OD) δ 27.72, 30.50, 40.81, 115.71, 115.82, 120.48, 127.29, 146.25, 149.98.

***N,N′*-1,2-Ethanediylbis(3,4,5-trihydroxybenzamide) Hydrate (10).** The ethylene derivative **10** was prepared from 3,4,5-trimethoxybenzoic acid (2.0 g) and ethylenediamine using the procedure described above. Recrystallization from MeOH/H_2O gave the desired product as a white solid (0.30 g, 16%): mp 230−231 °C; IR 3509, 3322, 1581, 1531, 1452, 1365, 1032 cm^{-1}; ^{1}H NMR (CD_3OD) δ 3.49 (4H, m), 6.98 (4H, s), 8.16 (2H, bs); ^{13}C NMR (CD_3OD) δ 39.04, 105.48, 123.95, 135.13, 144.20, 166.58.

***N,N′*-1,3-Propanediylbis(3,4,5-trihydroxybenzamide) Hydrate (11).** The propane derivative **11** was prepared from 3,4,5-trimethoxybenzoic acid (2.0 g) and 1,3-diaminopropane using the procedure described above. Recrystallization from H_2O (pH = 1) gave the desired as an off-white solid (0.50 g, 26%): mp 210−211 °C dec; IR 1594, 1523, 1308, 1179, 1041 cm^{-1}; ^{1}H NMR (CD_3OD) δ 1.76 (2H, bm), 3.39 (4H, m), 7.01 (4H, s), 8.13 (2H, bt); ^{13}C NMR (CD_3OD) δ 12.05, 34.60, 105.01, 123.75, 134.84, 144.04, 165.56.

***N,N′*-1,5-Pentanediylbis(3,4,5-trihydroxybenzamide) Hydrate (12).** The pentane derivative **12** was prepared from 3,4,5-trimethoxybenzoic acid (2.0 g) and 1,5-diaminopentane using the procedure described above. Recrystallization from MeOH/H_2O gave the desired product as a white solid (1.1 g, 58%): mp 210−211 °C dec; IR 1584, 1530, 1440, 1337, 1239, 1215 cm^{-1}; ^{1}H NMR (CD_3OD) δ 1.40 (2H, bm), 1.61 (4H, bm), 3.29 (4H, m), 6.97 (4H, s), 7.66 (2H, bt); ^{13}C NMR (CD_3OD) δ 23.25, 28.08, 38.61, 105.74, 124.63, 135.04, 144.25, 166.52.

***N,N′*-1,6-Hexanediylbis(3,4,5-trihydroxybenzamide) Hydrate (13).** The hexane derivative **13** was prepared from 3,4,5-trimethoxybenzoic acid (2.0 g) and 1,6-diaminohexane using the procedure described above. Recrystallization from MeOH/H_2O gave the desired product as a white solid (0.57 g, 28%): mp 210−211 °C dec; IR 1582, 1538, 1445, 1348, 1041 cm^{-1}; ^{1}H NMR (CD_3OD) δ 1.37 (4H, bm), 1.57 (4H, bm), 3.31 (4H, m), 6.98 (4H, s), 7.91 (2H, t, $J = 5.50$ Hz); ^{13}C NMR (CD_3OD) δ 24.85, 27.84, 37.81, 105.18, 124.15, 134.74, 144.01, 165.69.

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