Derivatives of Dexanabinol. I. Water-Soluble Salts of Glycinate Esters

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Purpose. Glycinate ester-type water soluble derivatives of dexanabinol (HU-211) (1) a non-psychotropic cannabinoid with potential use in the treatment of brain damage were synthesized and evaluated as prodrugs or congeners.

Methods. Conventional procedures were used for the synthesis of the novel derivatives. Stability studies in water and blood (rat, dog, human) were performed by HPLC; NMDA receptor binding was determined by radio ligand [^{3}H] MK-801-displacement; the neuroprotection and neurotoxicity studies were performed in cortical cell cultures.

Results. Glycinate (3), dimethyl- and diethylamine (5, 6), trimethyl- and triethyl- ammonium (7, 8) acetates of 1 were synthesized. All compounds were relatively soluble and stable in water. The quaternary ammonium salt-type derivatives rapidly hydrolyzed to the parent drug in various types of blood including human. *In vitro* activity studies indicated that the novel derivatives possess NMDA receptor binding properties. The neuroprotecting properties manifested by some of the new derivatives were associated with very low neuronal cell toxicity and are credited to parent compound released by hydrolysis during the experiments rather than to intrinsic activity.

Conclusions. Compounds 7 and 8 are promising water-soluble prodrug candidates for 1; the glycinate ester 3 might be used as an active analog.

KEY WORDS: dexanabinol; glycinate; cannabinoid; prodrugs.

INTRODUCTION

Dexanabinol (HU-211) (1), first synthesized by Mechoulam et al (1), is the (+) 3S, 4S enantiomer of (−) 3R,4R-5′(1′,1′-dimethylheptyl) 7-hydroxy-Δ^6-tetrahydrocannabinol (HU-210). While naturally occurring cannabinoids belonging to the (−) 3R, 4R series bind to specific central (3,4) and peripheral (5) cannabinoid receptors, the (+) 3S, 4S isomers have only negligible affinity to these receptors (6-8). Dexanabinol on the other hand is a noncompetitive N-methyl-D-aspartate (NMDA) receptor antagonist (9). As a result, in contrast to the (−) 3R, 4R isomer (HU-210) (a synthetic compound as well) which is one of the most potent cannabimimetic agents thus far described (1,2,10), 1 is devoid of any comparable activity (10,11).

As promising neuroprotective properties have been demonstrated in a number of *in vitro* and *in vivo* studies (9,12-16) the development of 1 as a therapeutic agent with potential use in the treatment of brain damage associated with stroke, cardiac arrest and trauma (17-19), has been initiated.

The amphipathic nature of 1 enables its access to the central nervous system, since it readily passes the blood-brain barrier. Unfortunately, the lipophilic character of 1 is also associated with very poor solubility in water. This makes the development of formulations suitable for intravenous administration of 1 difficult, hampering its clinical application and development. Water-soluble derivatives of 1 designed to readily release the drug by hydrolysis following i.v. administration might overcome this problem and could be used as prodrugs (20-22). In addition, derivatives with increased aqueous stability but which are hydrolytically stable could be used as easy-to-formulate analogs (congeners) if they possess significant intrinsic NMDA antagonist activity. So far no SAR studies in the (+) 3S, 4S series of cannabinoids have been reported.

Derivatives with increased aqueous solubility can be prepared by the attachment of polar or permanent charge bearing groups to the allylic (C-7) or phenolic (C-3′) hydroxyl functionalities of 1, the two sites suitable for reversible structural modifications, through carboxylic ester or phosphate linkages. The synthesis and evaluation of the glycinate and N-substituted glycinate esters attached to the allyic OH of 1 are reported herein.

MATERIALS AND METHODS

Melting points are uncorrected and were determined on an Electro-thermal melting point apparatus (Fisher Scientific). Elemental microcombustion analyses were performed by Atlantic Microlabs Inc., Atlanta, Georgia. Proton (^{1}H NMR) and carbon (^{13}C NMR) nuclear magnetic resonance spectra were recorded on a Varian XL-300 spectrometer. Samples were dissolved in an appropriate deuterated solvent and chemical shifts were reported as parts per million (δ) relative to tetramethylsilane (0.00) which served as an internal standard. Coupling constants (J) are reported in Hertz. (NMR data are presented in the appendix). Thin layer chromatography was performed on EM reagents DC-aluminum foil plates coated to a thickness of 0.2 mm with silica gel (60 mesh). All solvents and chemicals were reagent grade. Dexanabinol was obtained from the Casali Institute of Applied Chemistry, Hebrew University of Jerusalem, Israel.

7-(N-t-BOC-glycyl) dexanabinol (2). To a solution of dexanabinol (1.00 g, 2.59 mmol) in acetonitrile (20 mL) and dimethylformamide (5 mL) were added N-t-BOC- glycine (0.544 g, 3.10 mmol), dicyclohexylcarbodiimide (0.64 g, 3.10 mmol) and dimethylaminopyridine (0.025 g, 0.2 mmol). The reaction mixture was stirred under anhydrous conditions at 22 - 25°C for 3 days. The precipitated dicyclohexylurea was filtered, the solution was evaporated in vacuo and the residue was purified by chromatography. Silica gel: Aldrich, 200 - 425 mesh, 40 × 30 cm: eluted successively with hexane,

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2% and 4% ethyl acetate in hexane (200 mL each) and 6% ethyl acetate in hexane (1500 mL) to give 0.55 g (40%) of 2 (purity according to HPLC peak area: 99.3%). Recrystallization (nitromethane) of a second fraction (91% purity) afforded a total yield of 0.96g (70%) of pure compound (>99%), m.p. 110-112°C. Anal. Calcd for $C_{32}H_{49}O_6N$: C, 70.69; H, 9.08; N, 2.58. Found: C, 70.58; H, 9.08; N, 2.58.

7-Glycyl dexanabinol hydrochloride (3). A solution of 2 (0.35 g, 0.64 mmol) in 2 N hydrochloric acid in ethyl acetate (3 mL) was stirred at 20 - 25 °C for 10 min. The solvent was removed in vacuo and the residue slurried with ethyl ether (5 mL) and dried affording 0.28 g (90% yield) of 3 as an off-white solid; (purity according to HPLC peak area: 97.7%). Anal. Calcd for $C_{27}H_{42}ClNO_4$: C, 67.55; H, 8.82; Cl, 7.38; N, 2.91. Found: C, 67.40; H, 8.90; Cl, 7.55; N, 3.20. The high resolution FABMS calcd $[M-H]^+$: 444.31; found: 444.31.

7-Bromoacetyl dexanabinol (4). To a solution of 1 (0.100 g, 0.26 mmol) in toluene (6 mL) was added bromoacetic anhydride (0.118 g, 0.45 mmol) and the resulting mixture was stirred at 20 - 25 °C for 48 h. The organic solution was extracted with water (2 × 2 mL) then dried over Na_2SO_4 and evaporated. The residue was purified by chromatography (silica gel: Aldrich, Merck grade 60, 230 - 400 mesh, 32 × 2 cm; elution with hexane containing gradually increasing concentrations of ethyl acetate from 0 to 6%) yielding 0.123 g (93.4% yield) of 4 as an oil. Purity according the HPLC peak area: 96.8%. Anal. Calcd for $C_{27}H_{39}BrO_4$: C, 63.90; H, 7.75; Br, 15.74. Found: C, 63.68; H, 7.73; Br, 15.82.

7-Dimethylaminoacetyl dexanabinol (5). To a solution of 4 (0.119 g, 0.24 mmol) in hexane (6 mL) degassed with argon was added dimethylamine (gas) and the mixture was stirred for 2 h at 20 - 25° C. The precipitated dimethylamine hydrobromide was filtered off and the solution was evaporated to dryness and the resulting residue slurried with hexane (3 × 2 mL). After drying, 0.100 g (yield 90%) of 5 was obtained, m.p. 110 - 112 ° C; purity according to HPLC peak area: 99.6%. Anal. Calcd for $C_{29}H_{45}NO_4$: 0.25 H_2O C, 73.15; H, 9.63; N, 2.94. Found: C, 73.14; H, 9.73: N, 2.94.

7-Diethylaminoacetyl dexanabinol (6). To a solution of 4 (0.178 g, 0.34 mmol) in hexane (6 mL) degassed with argon was added diethylamine (2 mL, 1.41 g, 1.9 mmol) and the mixture was stirred for 4 h at 20 - 25°C. The precipitated diethylamine hydrobromide was filtered off, the solution was evaporated to dryness and the residue slurried with hexane (3 × 2 mL). After drying (temperature below 50°C), 0.160 g of 6 (yield 94%) resulted as an off white solid, m.p. 128 - 130°C; purity according to HPLC peak area: 98.4%. Anal. Calcd for $C_{31}H_{49}NO_4$: C, 74.51; H, 9.88; N, 2.80. Found: C, 74.40; H, 9.89; N, 2.82.

The hydrochloride salts 5a and 6a of 5 and 6 were prepared by adding a solution of HCl in ether to solutions of 5 or 6 in ether followed by filtering and rinsing the resulting solid with ether.

(Dexanabinol-7-acetyl)-trimethylammonium bromide (7). To a solution of 4 (0.81 g, 1.60 mmol) in hexane (15 mL) placed in a closed system under argon, trimethylamine (gas) was added at 20-25°C. The resulting mixture was stirred for 18 h at 20-25°C then the precipitate was filtered, and rinsed with hexane. After drying in vacuo, 0.89 g (yield 89%) of crude 7 was obtained. Purification (slurry with 3 × 15 mL cold ether) yielded 0.49 g (54.3%) of pure compound. Purity according to HPLC peak area: 98.1%, m.p. 178-180°C. Anal. Calcd for $C_{30}H_{48}BrNO_4$: C, 63.59; H, 8.54; Br, 14.10; N, 2.47. Found: C, 63.35; H, 8.53; Br, 14.03; N, 2.38.

(Dexanabinol-7-acetyl)-triethylammonium bromide (8). To a solution of 4 (0.081 g, 0.16 mmol) in toluene (45 mL) was added triethylamine (5 mL, 3.6 g, 0.035 mmol) and the mixture was stirred under argon atmosphere for 3 days at 20 - 25 °C. The precipitated salt was filtered off and the solution was evaporated to dryness. The residue was slurried with toluene (3 × 5 mL) to afford 0.092g (95% yield) of 8. Purity according to HPLC peak area: 94%. Anal. Calcd for $C_{33}H_{54}BrNO_4$: C, 65.11; H, 8.94; Br, 13.13; N, 2.30. Found: C, 65.26; H, 8.70; Br, 13.25; N, 2.38.

Stability

Twice distilled water (pH 5.6 - 5.7) and freshly-obtained plasma (rat, dog, human) were used as media for stability studies.

Sample Preparation and Incubation Conditions. Compounds were dissolved in water or 10% (vol/vol) ethanol:water to concentrations of 2.0 - 7.3 mg/mL and 20 μL of the resulting solutions were added to 180 μL of water or freshly collected plasma to final concentrations of 200 - 730 μg/mL. The mixtures were incubated at 37°C for 1 h and 24 h. The pH of the aqueous solution was controlled, if necessary, and found unchanged during experiments. Samples were then extracted by addition of acetonitrile (3 vol) followed by vortexing and immediate centrifugation (Ependorf centrifuge). Aliquots of the organic (upper) phase were analyzed by HPLC. Zero-time determinations were performed by extracting the solution with acetonitrile immediately following the solution preparation. Amounts of dexanabinol resulting by hydrolysis of the examined esters (as μg and %) were determined by using a calibration curve. The concentration range of the calibration curve was 0.100 - 500 μg/mL. Average values for 3 experiments were reported.

Analytical Methodology. High performance liquid chromatography (HPLC) was used for quantitative analysis of dexanabinol. The HPLC consisted of a Kontron 410 solvent delivery system, Kontron 430 UV detector (variable wavelength capable of measuring at 2 channels simultaneously), Kontron 450-data system. The column was Econosphere C_{18} (100 mm × 4.6 mm 3μm (Alltech) equipped with C_{18} column guard (Alltech). The mobile phase was: acetonitrile: pH 3 aqueous phosphate buffer (KH_2PO_4-H_3PO_4, 0.01 M): 60:40. Flow rate 0.7 - 1.5 mL/min. Detection and quantitation was performed at two wavelengths (232 and 280 nm) simultaneously. Retention time of dexanabinol was in the range 3.5 - 4.5 min. Standard curves were linear over the examined concentration range.

Activity Studies

Forebrain Membrane Preparation. Brains were removed from Sprague-Dawley rats within 5 min after decapitation. Membrane preparations were conducted according to a procedure previously described (14).

Preparation of Cortical Cultures. Cortical cells were

derived from Sprague-Dawley rats and cultured according to procedures previously described (14,15).

Neurotoxicity Studies. Cells at 10 days in culture were exposed to dexanabinol derivative or to an appropriate vehicle, in the presence (for protection experiments) or in the absence (for toxicity evaluation) of 1 mM N-methyl-D-aspartate (NMDA) and 300 μM of glycine, in medium without serum. Aqueous ethanol (0.1% the final concentration) was used as vehicle for all derivatives. Cell mortality was quantitatively assessed by measurement of the lactate dehydrogenase (LDH) levels of the extracellular medium 20 hrs after the incubation was started as described in the literature (16,23).

Radioligand Binding Studies. Binding of [^{3}H] (+)-5-methyl-10,11-dihydro-5H-dibenzo [a,d] cyclohepten-5,10-imine maleate ([^{3}H]MK-801) to rat forebrain membranes was conducted in the presence of 10 μM glutamate and 30 μM glycine. Membranes (100 μg protein) were suspended in 50 mM tris-acetate, pH 7.4 buffer and incubated with 10 nM [^{3}H] MK-801 either alone or in the presence of dexanabinol derivative. In these studies 100 μM of the esters were dissolved in a vehicle consisting of 0.077% aqueous ethanol and 0.023% emulphor in the final concentration. The reaction volume was 0.5 mL. Non-specific binding was determined in the presence of 100 μM N-[1-(2-thienyl)cyclohexyl] piperidine (TCP). After 2 hrs incubations at 25°C, the radioligand binding process was terminated by rapid filtration using Whatman grade GF/B filters (Aldrich) presoaked in 0.1% polyethyleneimine and washing the filters with 15 mL cold buffer. Filters were counted in scintillation fluid using a liquid scintillation counter. Data were analyzed using the iterative non-linear least squares curve fitting program LIGAND (24).

Hydrolysis of Dexanabinol Esters During the Assays. The stability of various esters during the assays was determined. For receptor binding studies, solutions (10 μM and 100 μM) of esters in the vehicle used for the studies were incubated for 2 h with the buffer used in the assays or with the buffer and rat forebrain membranes. For activity studies 10 μM of each compound in the appropriate vehicle was incubated for 20 h either with the medium used for experiments or in the presence of the culture. The amount of 1 was then determined by HPLC. In the case of the cell cultures, 1 was quantitated in the incubation medium, cell cytosol and cell membranes, as described above. The sums of 1 found in the three fractions were divided by the sum of 1 found in the same fractions after 0 h incubation of 10 μM of 1 with sister cultures. To determine 1 in cytosol, after removing the medium, cultures were incubated with water for 30 min then centrifuged. Content of 1 was determined in the supernatant and in the cell membranes (pellets) after extraction with acetonitrile (twice). In each case the presented results are an average (or in some cases lower and upper limits) of 3 - 5 experiments.

RESULTS AND DISCUSSION

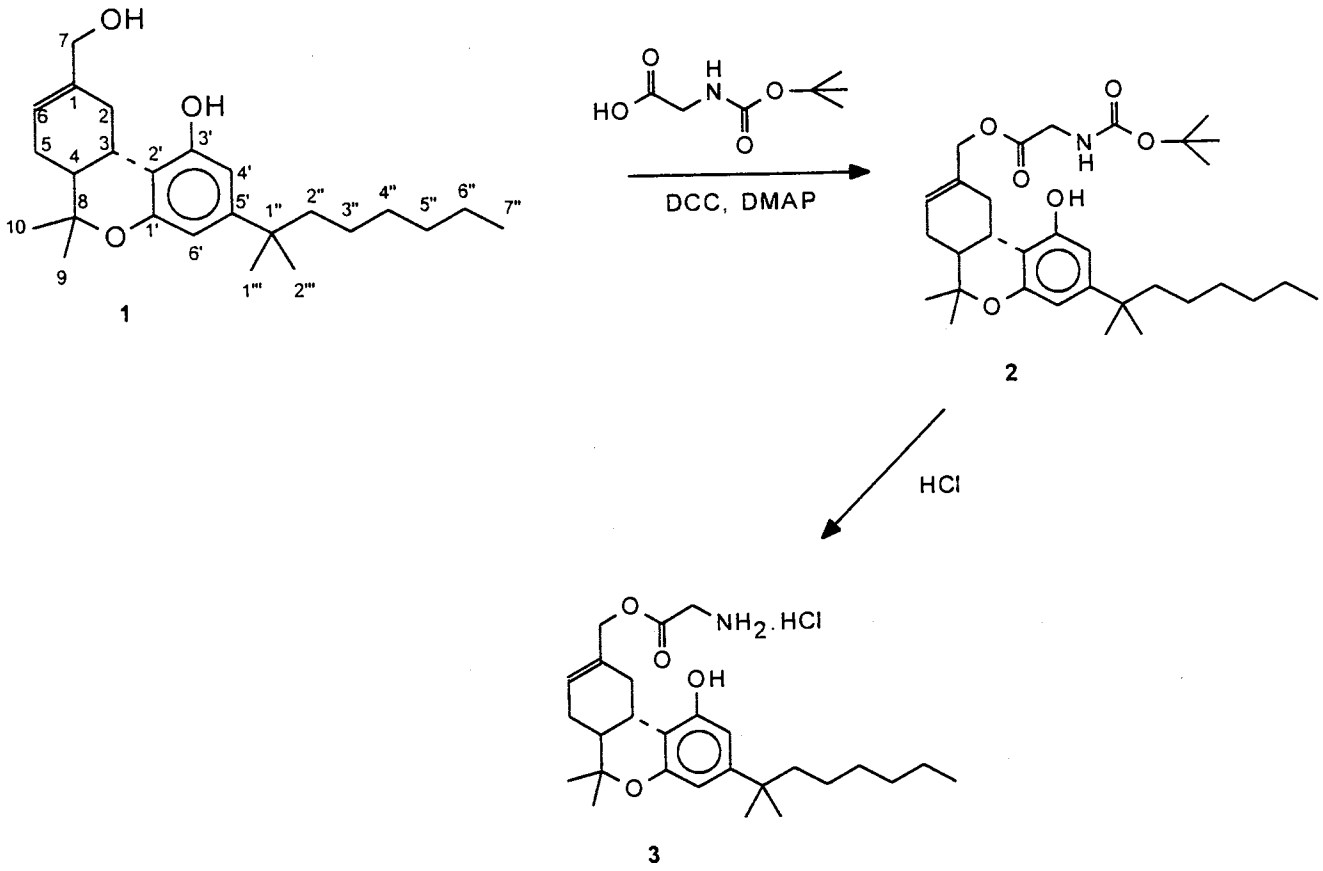
Chemistry

The allylic hydroxyl group at the C-7 position of dexanabinol is more reactive (chemically) than the sterically hindered and rigid phenolic C-3′ group. Due to this difference in reactivity, the acylation of 1 occurred preferentially at the allylic hydroxyl group, although small amounts of phenolic esters generally were present in the products. Various NMR techniques employed (proton, carbon, APT) support these structural assignments. The characteristic signal of the C-7 protons which appears at 3.95 ppm for dexanabinol was shifted to 4.5 - 4.6 ppm, characteristic for esters, in the new derivatives. In the ^{13}C NMR spectrum of the esters only the signals of C-1, C-6 and C-7 were shifted significantly as compared to 1 (from 139.7 ppm to ~133 ppm; from 121.6 ppm to 125 - 127 ppm; and from 67.2 ppm to 69 -70 ppm respectively) indicating structural modifications in the allylic region. Reaction of 1 with N-protected (t-BOC) glycine in a mixture of acetonitrile-dimethylformamide in the presence of dicyclohexylcarbodiimide (DCC) and 4-(dimethylamino)pyridine (DMAP) gave the 7-(N-t-BOC) glycyl ester of dexanabinol (2) which was purified by column chromatography. The t-BOC protecting group of 2 was removed by hydrochloric acid in ethyl acetate solution affording the HCl salt of the glycyl ester of dexanabinol (3) in pure form (Scheme 1). Several N,N-disubstituted derivatives of 3 were synthesized via the 7-bromoacetyl dexanabinol (4) which was obtained by acylation of 1 with bromoacetic anhydride in toluene followed by chromatographic column purification. Reaction of 4 with dimethylamine in hexane, produced the N,N-dimethylglycinate (5). The hydrochloride salt (5a) was obtained by the treatment of the free base with a solution of HCl in ether. In a similar manner, the N,N-diethylglycyl dexanabinol (6) and its hydrochloride salt (6a) were prepared. Two dexanabinol esters bearing permanent charges, the trimethylammoniumacetyl bromide 7 and the triethylammoniumacetyl bromide were obtained by reacting the 7-bromoacetyl derivative 4 with trimethylamine and triethylamine, respectively (Scheme 2).

Stability in Water and Susceptibility to Hydrolysis in Rat, Dog and Human Plasma

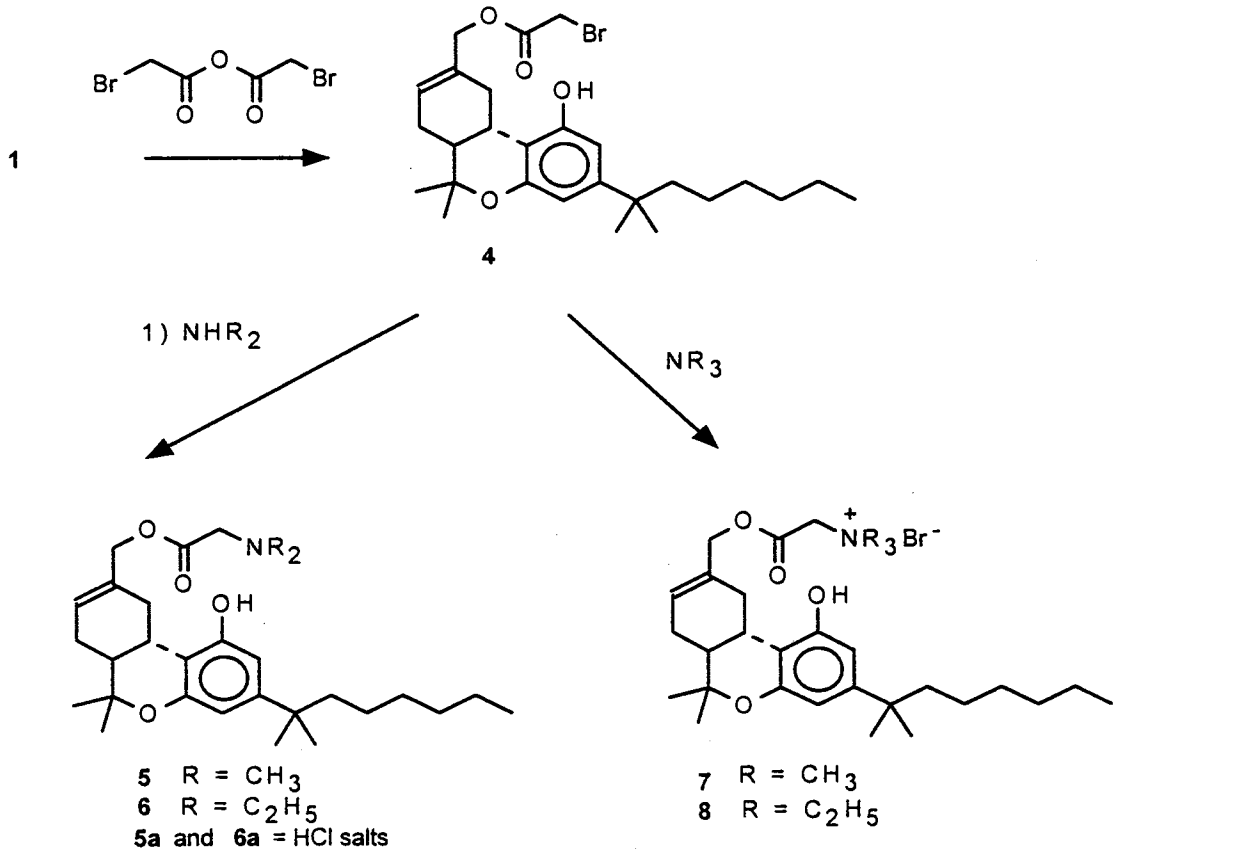
Prodrugs of practical use should have adequate solubility and sufficient stability to allow for formulation and storage (ideally in solution); they should rapidly (ideally spontaneously) convert to the active parent drug within the body. A preliminary stability study of the esters 3, 5a, 6a, 7 and 8 in water and plasma (from various sources) was performed in order to determine if these compounds can be used as prodrugs of 1. Dexanabinol which resulted by hydrolysis was quantitated by HPLC at zero-time and after 1 h and 24 h of incubation at 37°C in water and in plasma of rat, dog and human. Compounds 3, 7 and 8 are soluble in water (at least 2 - 7 mg/mL) while 5a and 6a are soluble in 10% (vol/vol) aqueous ethanol (when initially dissolved in ethanol and afterwards diluted with water).

The main role of the stability study in water has been to determine if degradation in plasma is enzymatic or via spontaneous hydrolysis. The results summarized in Table I indicate that all the examined esters are relatively stable in water; no spontaneous degradation was noticed for these compounds after 1 h and even after 24 h of incubation only of 0 - 7.5% of 1 was released by hydrolysis. Less than 0.5% of the glycinate 3 hydrolyzed after 1 h and less than 5% after 24 h; the N,N-dialkyl amino acetates 5a and especially 6a were



1 2 3

Scheme 1



1 4

5 R = CH_3
6 R = C_2H_5
5a and 6a = HCl salts

7 R = CH_3
8 R = C_2H_5

Scheme 2

Table I. Stability of Glycinate-Type Esters of Dexanabinol (HU-211)

Comp.	Time (h)	Water	Dexanabinol recovered %[a] plasma		
			Rat	Dog	Human
3	0	0.00	na	0.00	0.00
	1	0.40	na	0.88	0.95
	24	4.64	na	16.49	22.32
5	0	4.10	0.00	0.00	0.00
	1	4.28	10.82	0.00	5.15
	24	5.59	100.00	1.52	9.30
6	0	0.00	0.00	0.00	0.00
	1	0.00	15.68	0.74	0.00
	24	0.65	65.85	7.28	4.15
7	0	1.82	0.00	0.00	0.00
	1	1.88	86.00	95.54	91.92
	24	7.52	100.00	100.00	105.15
8	0	1.76	na	na	na
	1	3.64	50.20	57.90	67.70
	24	6.21	63.70	69.50	67.80

[a] Mean recovery ± max 10% SD.

also stable in water; the diethyl derivative was not hydrolyzed after 24 h; the two quaternary salts 7 and 8 were somewhat less stable in water but still only 6.2 - 7.5% of 1 was recovered after a 24 h incubation. These results are in agreement with recent theoretical findings, where the influence of various factors, including the nature of the acyl groups, on the hydrolysis of some cannabinoid esters, investigated in the framework of the AM1 molecular orbital approximation (25).

It is known that esterase activity and specificity and hence the stability of carboxylic esters in blood are species-specific (26). Esterases exist in multiple forms and might differ from species to species in type, activity-substrate concentration relationship and in substrate specificity. For this reason, a prodrug system which is optimized in rat or dog may not be useful in humans (27). In this study the hydrolytic stability of dexanabinol esters was determined in blood collected from rat, dog and human, with data obtained from human blood experiments most relevant. The glycinate salt (3) is relatively stable in all the examined media. The N,N-dimethylglycinate salt 5a is more stable than 3 in dog and human plasma. The N,N-diethylglycinate salt 6a is the most stable ester of the entire series, being only poorly hydrolyzed in dog and human plasma. Both 5a and 6a were less stable in rat plasma. A different profile was observed with the quaternary nitrogen containing compounds 7 and 8 (trimethylammonium- and triethylammoniumacetyl bromides respectively). Both of these compounds readily hydrolyzed in plasma of various sources including human. Of particular interest is compound 7 which rapidly hydrolyzed in human plasma (92% and 100% at 1 and 24 h respectively). The triethylammonium homolog 8 also appears to be completely hydrolyzed after 1 h, since the same amount of 1 (~ 68%) was recovered after both 1 h and 24 h of incubation.

Activity

Numerous studies preformed on the natural, (−) 3R, 4R cannabinoids have led to the establishment of qualitative and quantitative structure-activity relationships (QSAR) (2). However, these relationships do not necessarily apply to the (+) 3S, 4S series since, compounds having this configuration bind to NMDA rather than to cannabinoid receptors. It is not certain, for example, even if the allylic and phenolic hydroxylic functionalities are required for receptor recognition and/or binding of dexanabinol. Some data (Biegon, unpublished) suggest that this might not be the case. Prodrugs are by definition inactive combinations which are activated to release the parent drug following in vivo hydrolysis (20-22). However, based on the aforementioned considerations it was of interest to determine if the esters 3 - 8 possess intrinsic activity.

Three different assays were employed to determine the activity and toxicity of these combinations (14,16): (a) NMDA receptor binding as measured by the ability of the prepared ester to displace a known antagonist (b) in vitro protection of neurons against NMDA-induced toxicity and (c) neuronal cell toxicity potential. It has been demonstrated that dexanabinol inhibits the binding of [^{3}H]-TCP and [^{3}H]-MK-801, two noncompetitive NMDA antagonists, to the open state of the NMDA receptor channel (9,15). In vitro incubation of cortical neuron cultures extracted from fetal rat brains grown over a feeder layer of glial cells with 100-1000 μM NMDA resulted in death of 50 - 60% of the cells within 24 hrs. When dexanabinol, at a concentration of 1 - 10 μM was co-incubated with neurons exposed to NMDA, cell death was reduced or totally prevented in a dose dependent manner (14,16). This effect was similar in appearance and magnitude to the effect of the classical noncompetitive NMDA antagonist, MK-801. However, dexanabinol was not toxic to the host cells in these assays.

The dexanabinol esters 3, 5-8 were subjected to these tests. No enzymatic hydrolysis was expected to occur during receptor binding determinations given the protocol used. Non-enzymatic hydrolysis of the dexanabinol esters was determined after 2 h incubation at drug concentrations of 10 and 100 μM using the experimental buffer in the presence or absence of the rat forebrain membranes. The degree of hydrolysis was higher for 7 and 8 (3.0% and 8.4% in buffer and 11.3 and 10.7% in the presence of the forebrain, respectively), and lower (1.2 - 2.0 and 1.5 - 3.0, respectively) for the other esters (Table II). The dexanabinol concentrations after incubation were, in each case, too low to significantly inhibit [^{3}H] MK-801 binding. Consequently, the inhibition of [^{3}H] MK-801 binding is attributed to the esters themselves rather than to the dexanabinol released by hydrolysis. The data indicate that all compounds except for 6 have relatively good NMDA receptor binding properties, with IC_{50}'s varying between 6.8 and 20 μM as compared to 7.5 μM for 1, and the inhibition of [^{3}H] MK-801 binding to the NMDA receptor being 79 - 95% at a concentration of 100 μM. As discussed before, receptor binding appears to be an intrinsic property of these compounds.

Since, assays (b) and (c) required a longer incubation time, the hydrolysis of the esters during the experiments was more likely. The release of 1 from 10 μM solutions of various esters was determined after 20 h of incubation both in the medium used for experiments and in the presence of the cultures (Table III). In the first case compounds 5 and 6

Table II. Receptor Binding Properties of Dexanabinol Glycinate Esters

Comp.	Hydrolysis (% of 1 released) after 2 h of incubation: Buffer[a]	Hydrolysis (% of 1 released) after 2 h of incubation: Buffer + rat forebrain membranes[b]	Inhibition of [^{3}H]MK-801 binding[b]: av.IC_{50} (range) μM	Inhibition of [^{3}H]MK-801 binding[b]: % inhibition at 100 μM
1	—	—	7.5 (5–10)	92.5 (90.0–95.0)
3	2.0	1.5 (1.3–1.8)	13.8 (11.2–16.3)	90.5 (88.0–93.0)
5	1.6	3.0 (2.6–3.4)	17.5 (15.0–20.0)	85.0 (85.0)[a]
6	1.2	1.5 (1.5–1.6)	58.0 (58.0)	63.0 (63.0)[a]
7	11.3	3.4 (2.6–4.2)	8.2 (6.8–9.6)	83.5 (79.0–88.0)
8	10.7	8.4 (7.1–9.7)	17.8 (16.5–19.0)	88.0 (87.0–89.0)

[a] One determination.
[b] Mean values and range for 2–3 experiments.

released only 5 and 8% of 1 respectively, but these numbers increased to 59% and 24% in the presence of the cell culture components. The glycinate 3 and the two quaternary salt type combinations 7 and 8 both hydrolyzed in the medium (68 - 90%) and in the presence of the cultures (36 - 85%). This high degree of hydrolysis suggests that the neuroprotective activity determined for these combinations may be attributed to the released dexanabinol.

The neuroprotective activity results are collected in Table III. The glycinate ester 3 and the quaternary ammonium type derivatives, 7 and 8, most effectively protected neurons against NMDA mediated cell death, while at the same time, were devoid of cell toxicity (EC_{50}, 2 - 8 μM; 100% protection at 10 and even at 5 μM concentration). Compounds 5 and 6 proved to be less protective against NMDA toxicity; moreover 6 was toxic to neurons in 2 out of 3 experiments. These differences in the activity and toxicity of various glycinate esters could be attributed to different degrees of their hydrolysis and perhaps to some intrinsic properties of the unhydrolyzed compounds.

In summary, the results of this study indicate that the two quaternary ammonium type derivatives, the trimethyl- and triethyl- ammonium acetates, 7 and 8, respectively, are the most promising prodrug candidates among the examined glycinate esters. Derivatives 7 and 8 readily hydrolyze in human plasma, and are relatively soluble and stable in water. These compounds also have good receptor binding properties, manifest protection against NMDA induced toxicity, and are devoid of neuronal toxicity. The activity profiles of these compounds are most probably a result of activation of the parent 1 by hydrolysis of these enzymatically unstable esters. Thus, esters 7 and 8 appear to be good, water soluble prodrugs which should rapidly release dexanabinol after i.v. administration. As for the other examined conjugates: while 5a and 6a are too stable in human plasma to be used as prodrugs and their activity-toxicity profile is inferior to 1, the glycinate ester 3 might be used as an analog with increased solubility in water.

Table III. Activity of Dexanabinol Glycinate Esters

Comp.	Hydrolysis (% of 1 released) after 20 h of incubation[a]: Medium	Hydrolysis (% of 1 released) after 20 h of incubation[a]: Culture	EC_{50}[b] μM	Protection against NMDA toxicity % protection at: 5 μM	Protection against NMDA toxicity % protection at: 10 μM
1	—	—	3.8 (2.9–4.7)	92 (75–108)	100
3	68.0	40.0	5.0 (2.0–8.0)	89 (58–118)	na
5	8.0	59.0	na	21[c]	18[c]
6	5.0	24.0	na	41[c]	30[c]
7	90.0	85.0	3.5 (2.0–5.0)	99 (91–106)	100
8	77.0	55.0	5.0	100 (89–111)	100

[a] Mean value ± max 20% SD.
[b] Mean value and range for 3–5 experiments.
[c] One determiantion.

APPENDIX

NMR data for the novel derivatives: Compound (2). ^{1}H NMR ($CDCl_3$) δ: 6.26 (s, 1H, $C_{6'}$-H), 6.27 (s, 1H, $C_{4'}$-H), 5.78 (m, 1H, C_6-H), 5.78 (s, 1H, phenol OH), 5.12 (t-broad, 1H, J = 14.0, NH), 4.57 (s, 2H, C_7-H), 3.93 - 3.94 (d, 2H, J = 5.01, CO-CH_2), 3.40 - 3.34 (dd, 1H, J = 1.1; 16.7, $C_{2\alpha}$-H), 2.67 - 2.70 (m, 1H, C_3-H), 2.20 - 2.26 (m, 1H, $C_{5\beta}$-H), 1.45 (s, 9H, t-butyl CH_3s), 1.80 - 1.86 (m, 3H, $C_{2\beta}$-H, C_4-H, $C_{5\alpha}$-H), 1.41 - 1.52 (m, 2H, $C_{2''}$-H_2), 1.38 (s, 3H, C_9-H_3), 1.19 (s, 8H, $C_{3''}$-H_2, $C_{4''}$-H_2, $C_{5''}$-H_2, $C_{6''}$-H_2), 1.11 (s, 3H, C_{10}-H_3), 1.10 - 1.30 (s, 6H,$C_{1''}$-CH_3s), 0.84 (t, 3H, J = 6.4, $C_{7''}$-H_3). ^{13}C NMR ($CDCl_3$): δ 170.3 (NHCOO), 156.0 (OCO), 154.8 (C-3'), 154.3 (C-1'), 150.2 (C-5'), 133.3 (C-1), 124.9 (C-6), 109.6 (C-2'), 107.6 (C-6'), 105.6 (C-4'), 80.4 ($C(CH_3)_3$), 76.4 (C-8), 69.2 (C-7), 44.7 ($COCH_2$), 42.4 (C-4), 37.3 (C-1''), 31.8 (C-5), 31.6 (C-3), 31.2 (C-2), 30.0 (C-5''), 28.7 (C-1'''), (C-2'''), 28.3 ($C(CH_3)_3$), 27.5 (C-9), 24.6 (C-3''), 22.7 (C-6''), 18.4 (C-10), 14.10 (C-7''). Compound (3). ^{1}H NMR ($CDCl_3$): δ 8.36 (s, 3H, N^+-H_3), 6.42 (s, 1H, C_6,-H), 6.31 (s, 1H, $C_{4'}$-H), 5.66 (s, 1H, C_6-H), 4.51 (s, 2H, C_7-H), 4.10 (s, 2H, $COCH_2$), 3.30 - 3.40 (d, 1H, J = 15, $C_{2\alpha}$-H), 2.58 (s, 1H, C_3-H), 2.20 - 2.60 (m, 1H, $C_{5\beta}$-H), 1.70 (m, 3H, $C_{2\beta}$-H, C_4-H, $C_{5\alpha}$-H), 1.40 - 1.50 (m, 2H, $C_{2''}$-H), 1.32 (s, 3H, C_9-H), 1.20 (s, 8H, $C_{3''}$-H, $C_{4''}$-H, $C_{5''}$-H, $C_{6''}$-H), 0.98 - 1.30 (m, 6H, $C_{1''}$ CH_3s), 1.00 (s, 3H, C_{10}-H_3), 0.81 (t, 3H, J = 6.36, $C_{7''}$-H). ^{13}C NMR ($CDCl_3$); δ 168.2 (CO), 155.2 (C-3'), 154.0 (C-1'), 150.2 (C-5'), 132.7 (C-1), 126.3 (C-6), 109.4 (C-2'), 107.2 (C-6'), 106.2 (C-4'), 76.2 (C-8), 70.5 (C-7), 60.4 ($COCH_2$), 44.6 (C-4), 44.4 (C-2''), 37.2 (C-1''), 31.8 (C-5), 31.4 (C-3), 31.2 (C-2), 30.1 (C-5''), 29.7 (C-9), 28.8 (C-2'''), 28.5 (C-1'''), 27.5 (C-4''), 24.7 (C-3''), 22.7 (C-6''), 18.3 (C-10), 14.1 (C-7'''). Compound (4). ^{1}H NMR (DMSO-d_6): δ 9.22 (s, 1H, 3'-OH), 6.30 (s, 1H, $C_{6'}$-H), 6.14 (s, 1H, $C_{4'}$-H), 5.82 (s, 1H, C_6-H), 4.57 (d, 1H, J = 11.4,

C_7-H), 4.55 (d, 1H, J = 11.7, $C_{7\beta}$-H), 4.18 (s, 2H, $COCH_2$), 3.35 (dd, 1H, J = 1.2; 16.7, $C_{2\alpha}$-H), 2.50 - 2.62 (m, 1H, C_3-H), 2.12 - 2.28 (m, 1H, $C_{5\beta}$-H), 1.75 - 1.94 (m, 1H, $C_{2\beta}$-H), 1.50 - 1.74 (m, 2H, C_4-H, $C_{5\alpha}$-H), 1.36 - 1.50 (m, 2H, $C_{2''}$-H_2), 1.30 (s, 3H, C_9-H_3), 1.06 - 1.28 (m, 8H, $C_{3''}$-H_2, $C_{4''}$-H_2, $C_{5''}$-H_2, $C_{6''}$-H_2), 1.15 (s, 6H, $C_{1''}$-CH_3s), 1.00 (s, 3H, C_{10}-H_3), 0.80 (t, 3H, J = 6.4, $C_{7''}$-H_3). ^{13}C NMR: δ 153.8 (CO), 148.7 (C-5'), 132.9 (C-1), 124.6 (C-6), 109.2 (C-2'), 105.5 (C-6'), 104.9 (C-4'), 75.73 (C-8), 69.1 (C-7), 44.5 (C-4), 43,8 (C-2''), 39.2 (CO-CH_2), 36.8 (C-1''), 31.5 (C-5), 31.2 (C-3), 30.8 (C-2''), 29.4 (C-1'''), 28.7 (C-2'''), 27.3 (C-4''), 27.1 (C-9), 24.2 (C-3''), 22.1 (C-6''), 18.2 (C-10), 13.9 (C-7''). Compound (5). ^{1}H NMR ($CDCl_3$): δ 6.36 (s, 1H, $C_{6'}$-H), 6.26 (s, 1H, $C_{4'}$-H), 5.79 (s, 1H, C_6-H), 4.60 (d, 1H, J = 15.15, $C_{7\alpha}$-H), 4.57 (d, 1H, J = 15.4, $C_{7\beta}$-H), 3.31 (s, 2H, $COCH_2N$), 3.34 - 3.48 (d wide, 1H, $C_{2\alpha}$-H), 2.65 - 2.74 (m, 1H, C_3-H), 2.46 (s, 6H, $N(CH_3)_2$), 2.21 - 2.26 (m, 1H, $C_{5\beta}$-H), 1.81 - 1.92 (m, 3H, C_4-H, $C_{5\alpha}$-H, $C_{2\beta}$-H), 1.47 - 1.53 (m, 2H, $C_{2''}$-H), 1.39 (s, 3H, C_9-H_3), 1.21 (s, 8H, $C_{3''}$-H_2, $C_{4''}$-H_2, $C_{5''}$-H2, $C_{6''}$-H_2), 0.97 - 1.30 (m, 6H, $C_{1''}$-CH_3s), 1.10 (s, 3H, C_{10}-H_3), 0.85 (t, 3H, J = 6.5, $C_{7''}$-H_3). ^{13}C NMR ($CDCl_3$): δ 169.9 (CO), 155.1 (C-3'), 154.3 (C-1'), 150.1 (C-5'), 132.5 (C-1), 125.0 (C-6), 109.7 (C-2), 107.4 (C-6'), 105.7 (C-4'), 76.6 (C-8), 68.6 (C-7), 59.7 (COCH2), 44.9 ($N(CH_3)_2$), 44.7 (C-4), 44.5 (C-2''), 37.3 (C-1''), 31.8 (C-5), (C-3), 31.2 (C-2), 30.0 (C-5''), 28.7 (C-1'''), (C-2'''), 27.7 (C-4''), 27.5 (C-9), 24.6 (C-3''), 22.7 (C-6''), 18.4 (C-10), 14.1 (C-7''). Compound (6). ^{1}H NMR ($CDCl_3$): δ 6.38 (s, 1H, $C_{6'}$-H), 6.23 (s, 1H, $C_{4'}$-H), 5.78 (s, 1H, C_6-H), 4.50 (d, 1H, J = 15.0, $C_{7\alpha}$-H), 4.55 (d, 1H, J = 15.3, $C_{7\beta}$-H), 3.38 (s, 2H, CO-CH_2N), 3.32 - 3.46 (m, 1H, $C_{2\alpha}$-H), 2.70 (dd, 4H, J = 7.10; 14.3, N(CH_2-CH_3)$_2$), 2.62 - 2.78 (m, 1H, C_3-H), 2.20 - 2.30 (m, 1H, $C_{5\beta}$-H), 1.76 - 1.94 (m, 3H, C_4-H, $C_{2\alpha}$-H, C_{β}-H), 1.47 - 1.53 (m, 2H, $C_{2''}$-H_2), 1.39 (s, 3H, C_9-CH_3), 1.21 (s, 8H, $C_{3''}$-H_2, $C_{4''}$-H_2, $C_{5''}$-H_2, $C_{6''}$-H_2), 0.97 - 1.28 (m, 6H, $C_{1''}$-CH_3s), 1.11 (s, 3H, C_{10}-CH_3), 1.08 (5, 6H, J = 7.40, N(CH_2-CH_3)$_2$), 0.85 (t, 3H, J = 6.50, $C_{7''}$-H_3). ^{13}C NMR ($CDCl_3$): δ 171.3 (CO), 154.8 (C-3'), 154.4 (C-1'). 150.1 (C-5'), 133.6 (C-1), 124.7 (C-6), 109.7 (C-2'), 107.8 (C-6'), 105.6 (C-4'), 76.5 (C-8), 69.3 (C-7), 53.7 (CO-CH_2), 47.7 (N-(CH_2-CH_3)$_2$), 44.7 (C-4), 44.5 (C-2''), 37.3 (C-1''), 31.9 (C-5), 31.8 (C-3), 31.2 (C-2), 30.0 (C-5''), 28.8 (C-1'''), 28.7 (C-2'''), 27.7 (C-4''), 27.6 (C-9), 24.6 (C-3''), 22.7 (C-6''), 18.5 (C-10), 14.1 (C-7''), 12.2 (N(CH_2-CH_3)$_2$). Compound (7). ^{1}H NMR ($CDCl_3$): δ 6.51 (s, 1H, $C_{6'}$-H), 6.33 (s, 1H, $C_{4'}$-H), 5.78 (s, 1H, C_6-H), 5.12 (s, 2H, $COCH_2$), 4.69 (d, 1H, J = 11.3, $C_{7\alpha}$-H), 4.54 (d, 1H, J = 11.8, $C_{7\beta}$-H), 3.63 (s, 9H, $N(CH_3)_3$), 3.30 - 3.55 (m, 1H, $C_{2\alpha}$-H), 2.65 - 2.57 (m, 1H, C_3-H), 2.18 - 2.25 (m, 1H, $C_{5\beta}$-H), 1.64 - 1.96 (m, 3H, C_4-H, $C_{5\alpha}$-H, $C_{2\beta}$-H), 1.40 - 1.50 (m, 2H, $C_{2''}$-H_2), 1.37 (s, 3H, C_9-H), 1.60 (s, 8H,$C_{3''}$-H_2, $C_{4''}$-H_2, $C_{5''}$-H_2, $C_{6''}$-H_2), 1.11 (s, 3H, C_{10}-H), 0.97 - 1.28 (m, 6H, $C_{1''}$-CH_3s), 0.72 - 0.91 (t, 3H, J = 6.15, $C_{7''}$-H). ^{13}C NMR ($CDCl_3$): δ 164.4 (CO), 155.2 (C-3'), 153.9 (C-1'), 132.8 (C-1), 126.5 (C-6), 109.3 (C-2'), 107.0 (C-6'), 106.4 (C-4'), 76.2 (C-8), 70.0 (C-7), 63.0 ($COCH_2$), 54.3 ($N(CH_3)_3$), 44.9 (C-4), 44.4 (C-2''), 37.3 (C-1''), 31.8 (C-5), 31.3 (C-3), 30.6 (C-2), 30.0 (C-5''), 28.6 (C-1'''), 28.6 (C-2'''), 27.9 (C-4''), 27.4 (C-9), 24.6 (C-3''), 22.6 (C-6''), 18.3 (C-10), 14.1 (C-7''). Compound (8). ^{1}H NMR ($CDCl_3$): δ 6.62 (s, 1H, $C_{6'}$-H), 6.30 (s, 1H, $C_{4'}$-H), 5.80 (s, 1H, C_6-H), 4.70 - 4.78 (s, 2H, $COCH_2$), 4.69 (d, 1H, J = 11.0, $C_{7\alpha}$-H), 4.54 (d, 1H, J = 11.8, $C_{7\beta}$-H), 3.75 (m, 6H, $N(CH_2CH_3)_3$), 3.45- 3.54 (m, 1H, $C_{2\alpha}$-H), 2.57 - 2.65 (m, 1H, C_3-H), 2.18 - 2.25 (m, 1H, $C_{5\beta}$-H), 1.64 - 1.96 (m, 3H, C_4-H, $C_{5\alpha}$-H, $C_{2\beta}$-H), 1.40 - 1.50 (m, 2H, $C_{2''}$-H), 1.37 (s, 3H, C_9-H), 1.48 (s, 8H, $C_{3''}$-H_2, $C_{4''}$-H_2, $C_{5''}$-H_2, $C_{6''}$-H_2), 1.20 (s, 9H, $N(CH_2CH_3)_3$), 0.97 - 1.28 (m, 6H, $C_{1''}$-CH_3s), 0.80 - 0.90 (t, 3H, J = 6.15, $C_{7''}$-H_3). ^{13}C NMR($CDCl_3$): δ 164.1 (CO), 155.9 (C-3'), 153.7 (C-1'), 150.1 (C-5'), 132.8 (C-1), 127.2 (C-6), 109.1 (C-2'), 106.5 (C-6'), 106.3 (C-4'), 76.0 (C-8), 70.3 (C-7), 56.4 ($COCH_2$), 55.1 ($N(CH_2CH_3)_3$), 44.8 (C-4), 44.4 (C-2''), 37.3 (C-1''), 31.8 (C-5), 31.3 (C-3), 31.0 (C-2), 30.0 (C-5''), 28.7 (C-1'''), 28.6 (C-2'''), 27.98 (C-4''), 27.4 (C-9), 24.6 (C-3''), 22.6 (C-6''), 18.3 (C-10), 14.0 (C-7''), 8.3 ($N(CH_2CH_3)_3$).

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