Pergamon

Bioorganic & Medicinal Chemistry Letters, Vol. 7, No. 5, pp. 601-606, 1997

0960-894X/97 $17.00 + 0.00

PII: S0960-894X(97)00068-1

SYNTHESIS, MOLECULAR MODELING, AND BIOLOGY OF THE 1-BENZYL DERIVATIVE OF APDC–AN APPARENT mGluR6 SELECTIVE LIGAND

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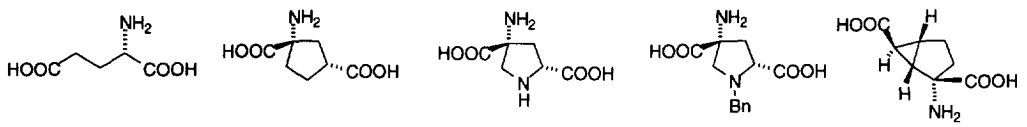
Abstract: The synthesis of the 1-benzyl derivative of (2*R*,4*R*)-4-aminopyrrolidine-2,4-dicarboxylic acid (1-benzyl-APDC) starting from *cis*-4-hydroxy-D-proline is disclosed together with a study of the activity of this compound at metabotropic glutamate receptors (mGluRs). The compound was found to display good mGluR6 selectivity, and may thus be a useful pharmacological research tool.

The amino acid glutamate plays a pivotal role in biological processes ranging from memory and learning to neuronal degeneration. This major excitatory amino acid (EAA) acts through disparate glutamate receptors, which can be categorized into two distinct types, the so-called ionotropic receptors and the metabotropic receptors.[1] The ionotropic glutamate receptors, or iGluRs, are associated with integral cation-specific ion channels and include the *N*-methyl-D-aspartate (NMDA), 2-amino-3-(5-methyl-3-hydroxyisoxazol-4-yl)propanoic acid (AMPA), and kainate subtypes. On the other hand, the metabotropic receptors are coupled to cellular effectors through GTP-binding proteins. The metabotropic glutamate receptors, or mGluRs, have been distinguished pharmacologically from the iGluRs by the use of the mGluR-selective agonist (1*S*,3*R*)-1-aminocyclopentane-1,3-dicarboxylic acid (1*S*,3*R*-ACPD) generally through measurements involving phosphoinositide hydrolysis or Ca^{2+} mobilization. To date the use of expression cloning techniques has led to the identification of eight mGluR subtypes, which have been placed into three major categories based on their molecular structure, signal transduction mechanisms, and pharmacological properties. Group I mGluRs (mGluR1 and 5) are coupled to phosphoinositide (PI) hydrolysis, whereas group II (mGluR2 and 3) and group III (mGluR4, 6, 7, and 8) are negatively linked to adenylyl cyclase activity. The group I receptors are more sensitive to quisqualic acid than they are to ACPD, the group II receptors are more sensitive to ACPD than quisqualic acid, and the group III receptors are most sensitive to 2-amino-4-phosphonobutyric acid (L-AP4).[2]

In order to better characterize the roles of GluRs in physiological processes,[3-5] there is an important need to identify novel, high affinity ligands that are family and subtype specific.[6-10] Toward this end, we became interested in exploring routes to heteroatom analogs of ACPD, and specifically both the nitrogen and oxygen compounds. While we had previously found little subtype selectivity for oxa-ACPD,[11] during the course of our efforts a report of the improved selectivity of the aza analog of ACPD, (2*R*,4*R*)-4-aminopyrrolidine-2,4-dicarboxylic acid or APDC, was disclosed by Schoepp and coworkers.[12-14] Additionally, a second report appeared by Tanaka and Sawanishi describing the synthesis of this compound from *cis*-4-hydroxy-D-proline.[15]

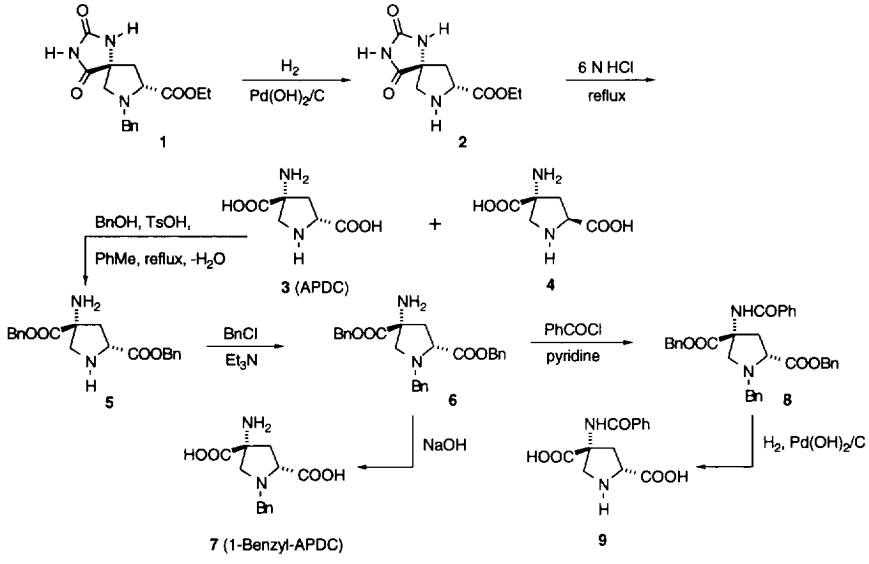


Based upon structural comparisons with other known mGluR ligands, we believed that the aza analogue of ACPD might be able to tolerate further substitution at its ring nitrogen. The preparation of the N-benzyl derivative and its biological selectivity for mGluR6 are described herein.

Chemistry. Our preparation of (2*R*,4*R*)-4-aminopyrrolidine-2,4-dicarboxylic acid (**3**) followed in its initial stages the procedure reported by Tanaka and Sawanishi[15] using *cis*-4-hydroxy-D-proline as the starting material, and we obtained the hydantoin **1** in good yield and optical purity.[16] We were, however, unable to

perform its hydrolysis under the conditions described by these authors (6 N HCl, sealed tube, 130 °C). The crude reaction mixture smelled of BnCl (indicating partial loss of the N-benzyl group) and displayed, after ion exchange to obtain the free amino acid, a ^{1}H NMR spectrum for the major component (that of our N-benzyl derivative **7**, see Scheme 1) distinctly different from the reported one. We initially concluded that the reaction temperature might have been too low, and that the product might possibly be a carboxamide due to partial

Scheme 1. Synthesis of APDC and 1-Benzyl-APDC.



hydrolysis of the hydantoin. Increasing the temperature to 150 and 165 °C resulted, however, in predominant and complete debenzylation and the formation of two products in a 7:10 and 1:1 ratio, respectively. The same 1:1 ratio of products was also obtained when the N-benzyl group was first removed hydrogenolytically and the resulting hydantoin **2** hydrolyzed at 180 °C. The failure of the reaction to proceed beyond this point argued strongly against the assumption of partial hydrolysis, and the two products displayed ^{1}H NMR spectra which were in reasonable agreement with Tanaka's and Sawanishi's spectra of **3** and its C-2 epimer **4**. These compounds evidently equilibrate in the higher temperature range. It was eventually found that **2** hydrolyzes without the need for pressure equipment in boiling 6 N HCl to give a mixture of mostly **3**, some **4**, and an unidentified byproduct, and that pure **3** crystallizes from a mixture of the free amino acids.[17]

The evaporated mother liquors from this hydrolysis reaction on esterification (BnOH, TsOH, PhMe, reflux, - H_2O) yielded, besides other products, small quantities of the dibenzyl ester **5** which served as a convenient starting material for N-substituted derivatives. The correct C-2 stereochemistry was established by hydrogenolysis (1 bar H_2, cat. 20% $Pd(OH)_2/C$, MeOH/H_2O 1:1, rt), which yielded the amino acid **3**. N-benzylation in analogy to the preparation of **1** (1.5 equiv. BnCl, 1.3 equiv. Et_3N, CH_2Cl_2; 63% of **6** + 12% of **5**) produced the 1-benzyl derivative **6** as a single regioisomer. That it is indeed the pyrrolidine nitrogen rather than the primary amino nitrogen that has undergone alkylation is not obvious but was shown by N-benzoylation (2 equiv. PhCOCl, pyridine, 0 °C to rt; 71%). The resulting monobenzoyl derivative **8** in C_6D_6 solution exhibited a broad singlet at δ 7.51 indicative of an amide proton, a result that requires the presence of an NH_2 group in the starting material. Compound **6** yielded the 1-benzyl derivative **7** of APDC on basic hydrolysis (5 equiv. 5 M aq. NaOH, MeOH, rt; 50%)[18] whereas **8** was transformed into the underlying amino acid **9** by hydrogenolysis (1 bar H_2, cat. 20% $Pd(OH)_2/C$, MeOH/H_2O 6:1, rt; 55%).[19]

Biological Results. Chinese hamster ovary (CHO) cells stably expressing mGluR1a, mGluR5a, mGluR2, or mGluR6 were cultured as described previously[20] and used for measurements of phosphoinositide (PI) hydrolysis or cAMP formation. For measurements of PI hydrolysis, cells expressing mGluR1a or mGluR5a were cultured in 24 well-plates and then labeled overnight with 1 μCi/mL of [^{3}H]*myo*-inositol

(specific activity 17 Ci/mmol, Amersham). CHO cells expressing mGluR2 or mGluR6 were cultured in 96-well plates. Measurements of PI hydrolysis and of forskolin-induced cAMP formation were performed as described previously.[21] The results of our studies employing ACPD, APDC, and 1-benzyl-APDC are shown in Table 1. Additionally, data are provided for the N-benzoyl derivative **9**.

Table 1. Selectivity of ACPD, APDC, 1-benzyl-APDC, and N-benzoyl-APDC **9** for the mGluR subtypes. (values are EC_{50}s for agonists, μM; antagonist activity is shown in parentheses as an IC_{50} value).[a]

Group	Receptor	ACPD	APDC	1-Benzyl-APDC	9
I	mGluR1 mGluR5	42 15	100 200	>1000 (IC_{50} = 600)	not active
II	mGluR2	5	0.3	(IC_{50} = 200)	not active
III	mGluR6	60	110	20	1000

[a]not active = no agonist or antagonist activity at a concentration of 1 millimolar.

Molecular Modeling. In order to obtain a better understanding of the conformational requirements for the binding of ligands to each mGluR subtype, molecular modeling studies were performed on ACPD, APDC, 1-benzyl-APDC, and L-glutamate (L-glu). A highly potent, selective mGluR ligand, LY354740,[22] was also included in the present study. All molecular modeling studies were conducted using the QUANTA program,[23] and all molecular mechanics and molecular dynamics simulations were performed using the CHARMM program.[24] In order to access the different conformations that may be adopted by each of these ligands, especially the ring conformations for ACPD, APDC, 1-benzyl-APDC, and LY354740, high temperature molecular dynamics simulations were performed at 1000 K for 50 picoseconds (ps). Trajectories were recorded every 0.1 ps and subsequently minimized for 1000 steps, or until convergence, defined as an energy gradient tolerance ≤ 0.001 kcal mol^{-1} $Å^{-1}$, using an adopted-basis Newton-Raphson algorithm as implemented in the CHARMM program.[24] The dielectric constant was set to 80 to simulate an aqueous environment. The carboxyl groups in these ligands were set to be deprotonated and the amine groups to be protonated, as expected under physiological conditions. These minimized structures were clustered using the Cluster Analysis module in the QUANTA program, using all nonhydrogen atoms as the reference atoms, to obtain truly distinct low energy conformations. In each conformational cluster, the lowest energy conformation was selected and compared with the distinct low energy conformations of L-glu. The comparisons were carried out using the Molecular Similarity module in the QUANTA program with the five carbon atoms and the nitrogen atom in L-glu serving as the reference atoms. This comparison essentially reveals the overall goodness of overlap between a low energy conformation of an mGluR ligand and a low energy conformation of L-glu. The root-mean-square (RMS) values were obtained for each ligand, which provide a quantitative measure of the goodness of the overlap.

L-Glu is a flexible molecule and possesses 9 local minima, as shown in Figure 1, with respect to its two torsion angles (Tor 1, C1-C2-C3-C4; Tor 2, C2-C3-C4-C5). Our calculations using the CHARMM program showed that these 9 conformations have an energy difference within 2.5 kcal/mol (Figure 1). The most stable conformation is the g^+g^+ (g = gauche; a = anti), which is also the conformation assumed in the crystalline state. The 5-membered ring structure of ACPD was found to adopt three distinctive conformations in our studies. Both APDC and 1-benzyl-APDC adopt two distinct low energy conformations. LY354740 also adopts two distinct conformations, although the difference between these two conformations is small (RMS = 0.23 Å). The distinct low energy conformations of ACPD, APDC, 1-benzyl-APDC, and LY354740 are shown in Figure 2.

Each of these distinct low energy conformations were superimposed on those of L-glu, and the results are summarized in Table 1. The energy differences among the three distinct conformations of ACPD are less than 1.0 kcal/mol. The superposition of ACPD on L-glu showed that ACPD overlaps nicely with the ag^+ and g^+a conformations of L-glu (RMS = 0.29 Å and 0.21 Å, respectively); also it overlaps fairly well with the aa, ag^-, and g^+g^+ conformations. ACPD, however, has a very poor overlap with the g^-g^+ conformation (RMS = 1.03 Å) and a relatively poor overlap with the g^+g^- and g^-g^- conformations (RMS = 0.79 Å in both cases), suggesting that these conformations are unlikely to be the desired conformation for its binding to mGluR2, since it displays a fairly good potency at this subtype. APDC is a more selective mGluR ligand than ACPD. It displays an EC_{50}

value of 0.3 μM to mGluR2 and more than 100 μM to mGluR1, mGluR5, and mGluR6. The 5-membered ring in APDC adopts two distinct conformations, the energy difference between which is less than 1 kcal/mol. The superposition of APDC on the nine conformations of L-glu showed that APDC has a good overlap with the g^+a conformation. It is of interest to note that APDC has an improved overlap with the aa conformation (RMS =

Table 1. Results of superposition of mGluR ligands on the 9 conformations of L-glutamate. The root-mean-square (RMS) values were obtained by superpositioning the distinct low energy conformations (as defined in Fig. 2) of each ligand on L-glu, using the 5 carbon atoms and one nitrogen atom in L-glu as the reference atoms in rigid-body fitting operations. For each ligand, only the lowest RMS value (Å) is provided.

Conformation	ACPD	APDC	1-Bn-APDC	LY354740
L-glu (aa)	0.52 (III)	0.41 (I)	0.44 (I)	0.17 (I)
L-glu (ag^+)	0.29 (III)	0.69 (I)	0.64 (II)	0.59 (I)
L-glu (ag^-)	0.55 (II)	0.69 (I)	0.61 (II)	0.74 (I)
L-glu (g^+a)	0.21 (I)	0.35 (II)	0.37 (I)	0.63 (I)
L-glu (g^+g^+)	0.57 (I)	0.52 (II)	0.58 (II)	0.98 (I)
L-glu (g^+g^-)	0.79 (I)	0.89 (II)	0.90 (I)	1.04 (I)
L-glu (g^-a)	0.68 (I)	0.73 (II)	0.73 (I)	0.57 (II)
L-glu (g^-g^+)	1.03 (I)	1.09 (I)	1.05 (I)	0.83 (II)
L-glu (g^-g^-)	0.79 (III)	0.95 (I)	0.97 (I)	0.78 (II)

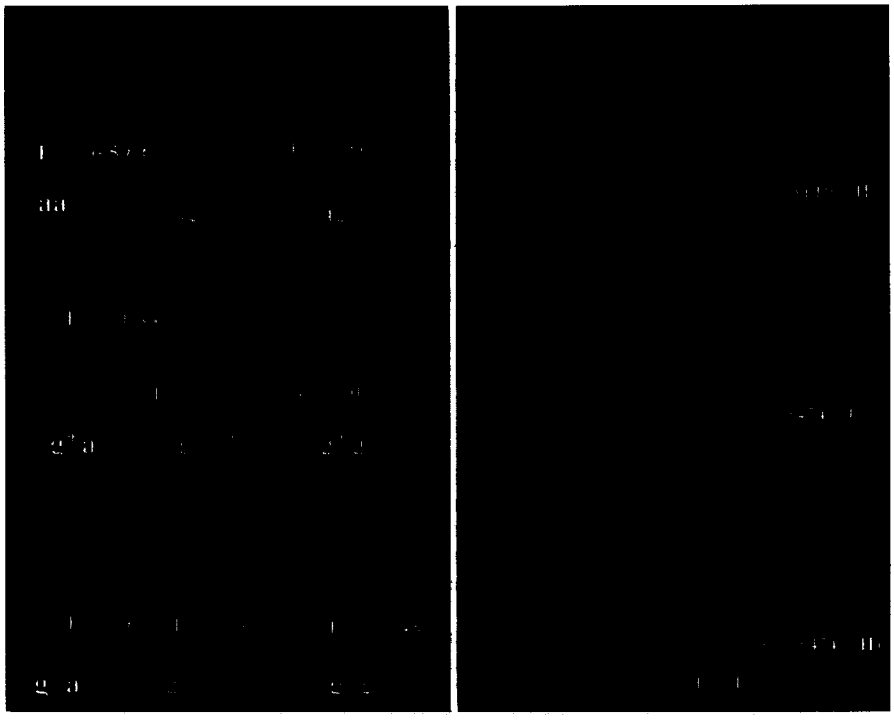


Figure 1. Nine local minima identified for L-Glu.

Figure 2. Low energy conformations found for ACPD, APDC, 1-benzyl-APDC, and LY354740.

0.41 Å) as compared to ACPD (RMS = 0.52 Å). It has poor overlap with the g^+g^-, g^-g^+, and g^-g^- conformations of L-glu, indicating that these conformations are unlikely to be the ones required for the binding of L-glu to mGluR2, as this ligand displays a high potency for mGluR2. 1-Benzyl-APDC adopts two distinct conformations with respect to its 5-membered ring that are very similar to those of APDC. As a consequence, 1-benzyl-APDC has a very similar profile to APDC when superimposing the respective conformations on the nine local minima of L-glu. Therefore, the decrease in the group I activity of 1-benzyl-APDC and the dramatic reduction in its mGluR2 activity suggest that space to accommodate the benzyl group is not available at group I receptors and at mGluR2. On the other hand, the improvement in its mGluR6 activity over that of ACPD and APDC indicates that this receptor can tolerate the additional steric bulk. It is possible that additional binding elements may exist on mGluR6 within this region.

LY354740 is a potent, group II selective ligand with an EC_{50} value of 35 nM at mGluR2.[22] The superposition of LY354740 on L-glu showed that this ligand has an excellent overlap with the aa conformation (RMS = 0.17 Å) and much poorer overlap with the other conformations. The fact that this ligand displays potent activity at mGluR2 strongly suggests that the aa conformation of L-glu may be the desired conformation for binding to mGluR2. This conclusion is also in good agreement with the results obtained for ACPD, APDC, and 1-benzyl-APDC.

In summary, our molecular modeling studies indicate that the aa conformation of L-glu is likely the desired conformation for the binding to mGluR2. It is also clear that a number of conformations of L-glu, such as g^-g^+ and g^-g^-, may be irrelevant for binding to mGluR1, mGluR5, mGluR2, and mGluR6. In addition, our results suggest that steric bulk of the benzyl group in 1-benzyl-APDC interferes with group I and mGluR2 activity, but not with mGluR6 activity. Thus, it may prove valuable to examine other modifications to this region of the ligand to further enhance mGluR6 selectivity and potency.

Discussion. As is apparent from Table 1, ACPD has little selectivity among the three groups of mGluRs. APDC, on the other hand, shows excellent selectivity for group II receptors, exhibiting an EC_{50} of 0.3 μM at rat mGluR2. This compares favorably with the result reported by Schoepp et al. in which they found an EC_{50} for inhibition of forskolin-stimulated cAMP formation of 3.5 μM in human mGluR2 expressing cells.[12] The N-benzoyl derivative of APDC, compound **9**, is inactive. This result underscores the important role that the basic, non-ring nitrogen atom of APDC plays in receptor recognition. Of particular note is the result found for the 1-benzyl derivative of APDC (**7**). While exhibiting very weak antagonist activity at group I and II, the compound is most potent and selective as an agonist for mGluR6. Although **7** is less potent than AP4 (EC_{50} = 1 μM at mGluR6), it is likely to exhibit other important properties, such as better bioavailability due to the presence of the lipophilic benzyl group, that should contribute to its usefulness as a pharmacological research tool (log P = 0.50 versus -2.26 for AP4). The present modeling studies, while somewhat limited by the availability of subtype selective ligands, appear to suggest that the aa conformation of L-glu is relevant to mGluR2 recognition. The modeling studies also suggest that the benzyl group in 1-benzyl-APDC sterically interferes with group I and mGluR2 binding, but is favorable to mGluR6 binding. This information may facilitate the design of other structurally novel mGluR ligands with improved potency and subtype selectivity.[25]

Acknowledgments. We are indebted to Pharmacia-Upjohn for their support of these studies. JTW also wishes to acknowledge NIH grant NS01720 for a research career development award.

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16. $[\alpha]_D$ +64.5°, $[\alpha]_{546}$ +76.8° (*c* 13.5 gL^{-1}, MeOH); ref 15 reports $[\alpha]_D$ +62.9° (*c* 11 gL^{-1}, MeOH). Mp 105.5-107.5 °C (ref 15: mp 105-106 °C).
17. Experimental procedure: A solution of 1.81 g (5.7 mmol) of **1** in 114 mL of MeOH was hydrogenated under 1 bar of H_2 over 62 mg of 20% $Pd(OH)_2/C$ (50% moisture) for 2 h. TLC (SiO_2, CH_2Cl_2/MeOH 11:1) confirmed completion of the reaction. The mixture was filtered over cotton and evaporated. The residue was taken up in 36 mL of 6 N aqueous HCl, and the solution was refluxed under N_2 (bath 120 °C) for 27.5 h. After evaporation, the residue was taken up in water and applied on a Dowex 50WX8 (H^+ form) ion exchange column. The column was eluted with water, then 1.5 M aqueous NH_3, then 3 M NH_3, and the progress of the elution was followed by TLC (SiO_2, isopropanol/water/conc. NH_3 4:1:1). Product-containing fractions were evaporated to dryness, the residue was dissolved in 10 mL of water, and a total of 15 mL of ethanol was added gradually with vigorous stirring; the mixture was seeded as soon as a permanent turbidity appeared. After standing at rt overnight, the precipitate was isolated by suction filtration, washed with ethanol/water 3:2, and dried in vacuo to obtain 0.48 g (48%) of **3** as a light-tan powder: ^1H NMR (D_2O, $Me_3Si(CH_2)_3SO_3Na$) δ 4.49 (t, 1 H, *J* = 8.5 Hz), 3.91, 3.65 (ABq, 2 H, *J* = 13 Hz), 2.95, 2.38 (ABq, 2 H, *J* = 16 Hz, A and B parts d, *J* = 8.5 Hz each); ^{13}C NMR (D_2O, internal $Me_3Si(CH_2)_3SO_3Na$) δ 175.69, 175.63, 65.55, 63.87, 54.06, 40.50; $[\alpha]_{546}$ +47.0°, $[\alpha]_{546}$ +56.2° (*c* 6.7 gL^{-1}, H_2O). The product of another run had $[\alpha]_D$ +47.8° (*c* 6.7 gL^{-1}, H_2O). These values are in good agreement with that reported by the Eli Lilly group in their full paper[13] ($[\alpha]_D$ +46.3° (*c* 1.0 gL^{-1}, H_2O)) but different from those published in the Eli Lilly patent ($[\alpha]_D$ +93.16°, no conditions given)[14] and ref. 15 ($[\alpha]_D$ -24.8° (*c* 6.5 gL^{-1}, H_2O)).
18. Colorless glass; ^1H NMR (D_2O, referenced to HDO, δ = 4.80) δ 7.41 (narrow m, 5 H), 4.14, 3.83 (ABq, 2 H, *J* = 12.5 Hz), 3.62 (t, 1 H, *J* = 8.5 Hz), 3.27, 3.20 (ABq, 2 H, *J* = 11.5 Hz), 2.81 (dd, 1 H, *J* = 9.5, 14.5 Hz), 2.08 (dd, 1 H, *J* = 7.5, 14 Hz); $[\alpha]_D$ +52° (*c* 1.3 gL^{-1}, H_2O).
19. Colorless crystals; mp (dec) 205-210 °C (from H_2O); ^1H NMR (D_2O, referenced to HDO, δ = 4.80) δ 7.76 (d, 2 H, *J* = 7 Hz), 7.63 (t, 1 H, *J* = 7 Hz), 7.52 (t, 2 H, *J* = 7.5 Hz), 4.47 (dd, 1 H, *J* = 7.5, 9 Hz), 4.18, 3.81 (ABq, 2 H, *J* = 12.5 Hz), 2.92, 2.75 (ABq, 2 H, *J* = 14 Hz, A part d, *J* = 9 Hz, B part d, *J* = 7 Hz); ^{13}C NMR (D_2O, external $Me_3Si(CH_2)_3SO_3Na$) δ 176.63, 175.07, 174.09, 135.43, 135.10, 131.47, 130.17, 66.98, 62.30, 55.33, 40.41; $[\alpha]_D$ +77°, $[\alpha]_{546}$ +92° (*c* 1.9 gL^{-1}, H_2O).
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(*Received in USA* 10 *December* 1996; *accepted* 24 *January* 1997)