Synthesis, Characterization, and Antimalarial Activity of the Glucuronides of the Hydroxylated Metabolites of Arteether

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The hydroxylated metabolites (log *P* 2.6–2.7) of β-arteether (**1**) in rat liver microsomes that retain their endoperoxide moiety showed comparable *in vitro* antimalarial activity to that of the parent drug arteether (log *P* = 3.89). The search for analogs of artemisinin (**7**) more suitable for intravenous use led to the synthesis of the glucuronide conjugates of the phase I hydroxylated metabolites of arteether which were found to have good water solubility, yet retained moderate lipophilicity (log *P* = 0.6–1.8). While a strong correlation was observed between the log *P* value of the glucuronides, the phase I metabolites, and the parent compound, it was found that 9β-hydroxyarteetherglucuronide (**26**) was the most active and the most polar (log *P* = 0.61) of the glucuronides. While the *in vitro* antimalarial activity of **26** (IC_{50} = 89.3 ng/mL) was found to be much less than that for the parent compound, the activity of **26** was within a range that would have potential therapeutic use.

Introduction

β-Arteether (**1**), the ethyl ether derivative of the natural product artemisinin (**7**), active principle of the Chinese medicinal plant *Artemisia annua* Linn,[1] is under investigation by WHO as a new antimalarial drug for its high level of blood schizontocidal activity against chloroquine resistant *Plasmodium falciparum* malaria[2] and especially cerebral malaria.[3] This novel antimalarial, isolated in 1972, has the structure of a sesquiterpene lactone with an internal peroxide linkage. The search for more potent analogs of artemisinin with better bioavailability has led to the synthesis of various semisynthetic derivatives.[4,5]

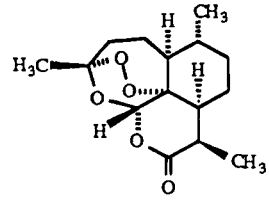
The metabolic pathway for arteether (**1**) in rat liver microsomal homogenates[6] consists of primarily O-dealkylation giving dihydroartemisinin (**2**) and hydroxylations at several sites giving 9β-hydroxyarteether (**3**), 9α-hydroxyarteether (**4**), 2α-hydroxyarteether (**5**), and 14-hydroxyarteether (**6**) (Figure 1).[7] Phase I transformations of this type produce metabolites that are more polar than the parent drug, and phase II reactions (conjugation pathways) would usually complete the detoxification of the drug.

While most drugs show best systemic activity in the moderate lipophilicity range (log *P* = 1.5–3.0),[8] arteether has been found to be extremely lipophilic (log *P* = 3.99). Hydroxylation of arteether results in compounds with lower log *P* values (estimated log *P* ~ 2.6) in the region optimal for systemic activity. This could explain the increased *in vitro* activity of some of the hydroxylated metabolites (with intact endoperoxide moiety) over that of arteether itself.[9] The glucuronides of the hydroxylated metabolites of arteether would still be fairly lipophilic (estimated log *P* values of ~1.5) and might be expected to retain reasonably good antimalarial activity with properties more suited for intravenous use. Other examples of glucuronides exhibiting significant biological activity are morphine-6-glucuronide (analgesic activity[10]) and benzidine-*N*-glucuronide (bladder cancer[11]).

The primary objective of the present study was to prepare and evaluate several glucuronide conjugates of arteether. β-Glucuronide of α-dihydroartemisinin (**17**), β-glucuronide of β-dihydroartemisinin (**18**), β-glucuronide of 9β-hydroxyarteether (**26**), β-glucuronide of 2α-hydroxyarteether (**27**), β-glucuronide of 14-hydroxyarteether (**28**), and β-glucuronide of 9α-hydroxyarteether (**29**) were synthesized chemically, starting from the hydroxylated metabolites which in turn were obtained chemically or using fermentation techniques. The lipophilicity of these compounds was determined experimentally and compared to the estimated or calculated values. The *in vitro* antimalarial activity of these standards was also evaluated.

Results

Chemistry. (A) Preparation of the Hydroxylated Metabolites of Arteether. Dihydroartemisinin (**2**) was obtained from artemisinin (**7**) by reduction of the lactone and further converted to arteether (**1**) using a modified procedure of Brossi et al.[12] Arteether was used as a substrate for microbial fermentation to obtain the hydroxyarteethers. Using *Cunninghamella elegans*, 9β-hydroxyarteether (**3**) was obtained in higher yield (40% as compared to the previously reported[7] 2% yield), by terminating the incubation at 6 days rather than the much longer (14 days) incubation used earlier. Using fermentation with *Streptomyces lavendulae* as previously reported,[7] arteether was also used to prepare 9α-hydroxyarteether (**4**), 2α-hydroxyarteether (**5**), and 14-hydroxyarteether (**6**). The products were purified by column chromatography, and structural conformation was accomplished by comparison of their ^1H- and ^{13}C-NMR[13,14] as well as by thermospray LC/MS data.[7]



7 Artemisinin

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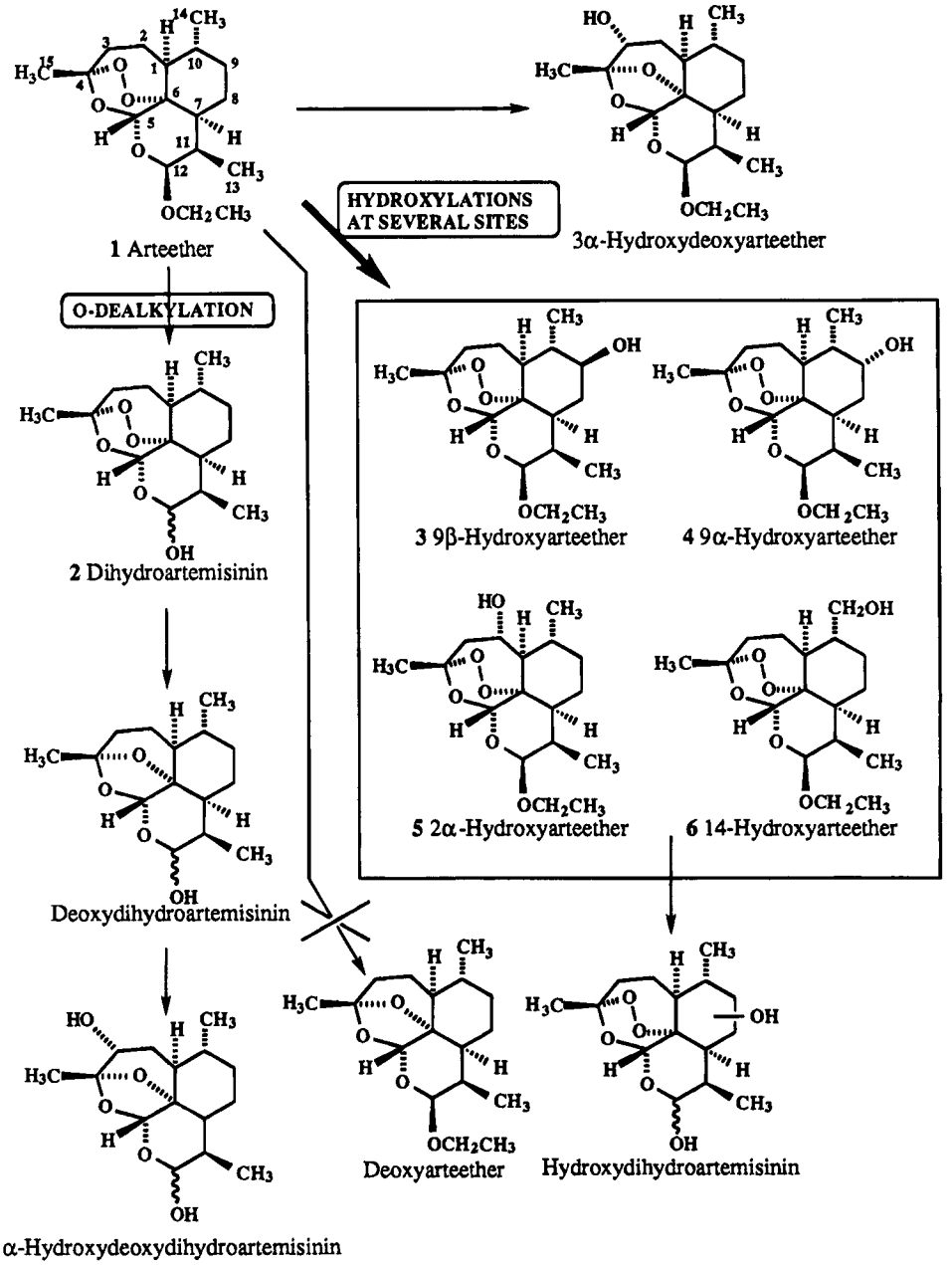
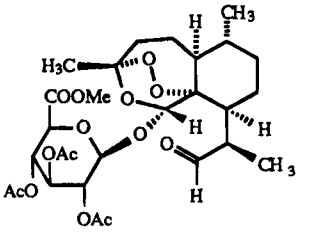
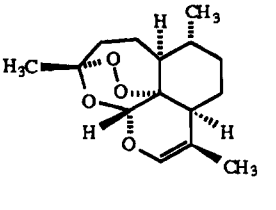


Figure 1. Mammalian metabolites of arteether in rat liver microsomes.

(B) Synthesis of the Glucuronide Tri-OAc Ester Derivatives of Dihydroartemisinin. Literature studies indicated two major pathways for the synthesis of the glucuronide tri-OAc ester derivatives.[15] Using the classical Koenigs–Knorr reaction[16–18] and condensing dihydroartemisinin (**2**) (a mixture of both α- and β-isomers in solution) with acetobromo-α-D-glucuronic acid methyl ester (α-bromo sugar) (**8**) in the presence of Ag_2CO_3 as catalyst at room temperature in benzene as a solvent (Figure 2) gave almost exclusively β-glucuronide (tri-OAc ester) of α-dihydroartemisinin (**10**) in low yields with minor amounts of α-glucuronide (tri-OAc ester) of β-dihydroartemisinin (**11**) and α-glucuronide (tri-OAc ester) of 11-epi-12β-dihydroartemisinin (**12**). Small amounts of an aldehydic (tri-OAc ester) byproduct (**15**) and anhydrodihyroartemisinin[19] (**16**) were also formed. In order to improve yields, molar ratios of both sugar and the Ag_2CO_3 catalyst were optimized and added in portions. The formation of β-glucuronide (tri-OAc ester) of β-dihydroartemisinin (**13**) was not observed using this procedure.



15 Aldehydic byproduct



16 Anhydrodihydroartemisinin

While the above classical Koenigs–Knorr method had afforded β-glucuronide (tri-OAc ester) of α-dihydroartemisinin (**10**), the target compound **13** required the development of an alternate synthesis. The use of

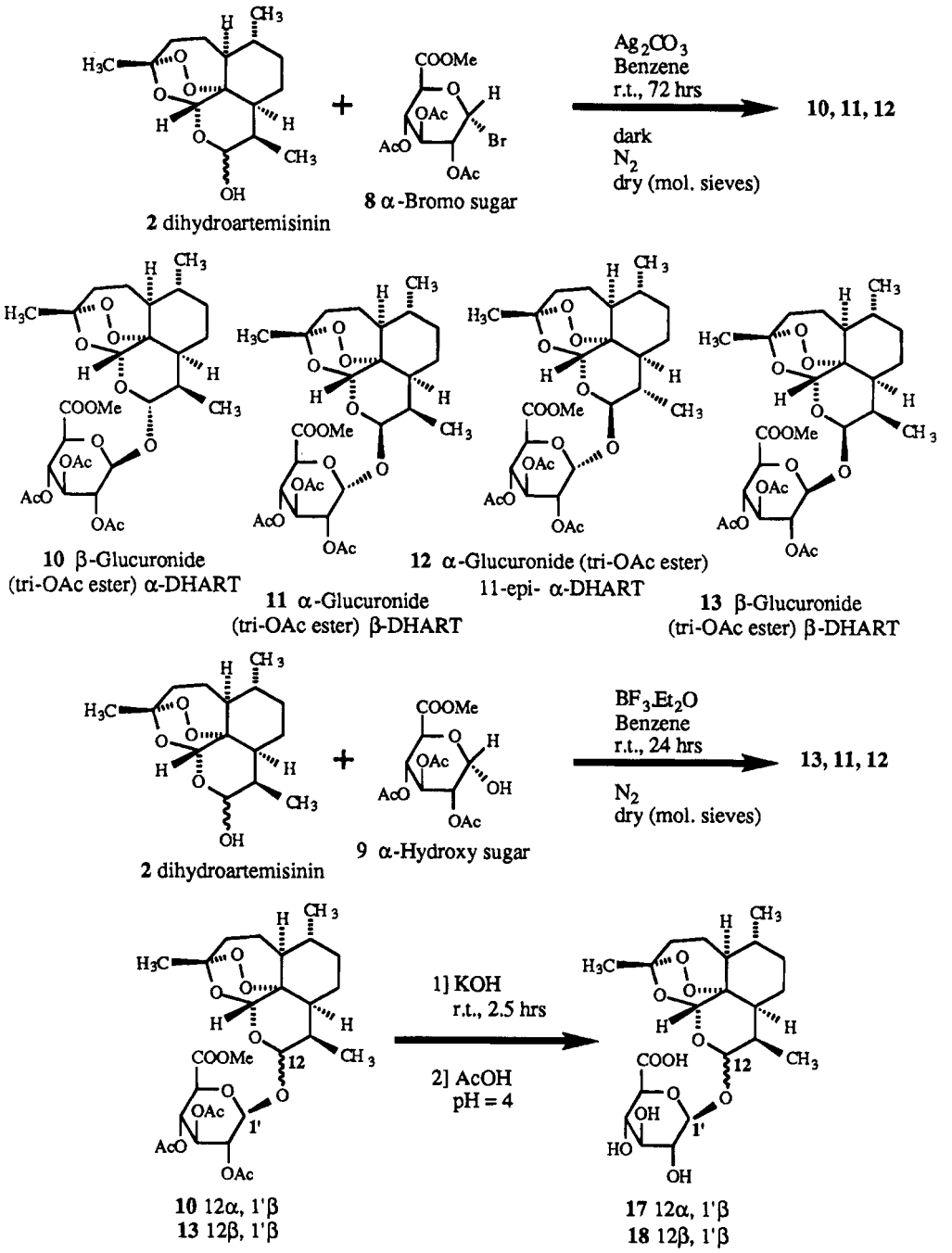


Figure 2. Chemical synthesis of glucuronide tri-OAc ester derivatives of dihydroartemisinin.

α-bromo sugar **8** and $CdCO_3$ or $Hg(CN)_2$ as catalysts or the use of methyl 1,2,3,4-tetra-*O*-acetyl-β-D-glucuronate (β-tetra-OAc sugar) (**14**) and $SnCl_4$ or *p*-TsOH as a catalyst[15] resulted in decomposition of the sugar and dihydroartemisinin (giving deoxyartemisinin[20]). Arteether has been synthesized by condensing dihydroartemisinin with a large excess of ethanol and catalytic amounts of $BF_3{\cdot}Et_2O$ in ~75% yield.[12] Since dihydroartemisinin was stable to $BF_3{\cdot}Et_2O$, investigations were conducted in the use of a catalytic amount of this reagent with the sugar in the α-configuration to form the remaining target compound. Condensation of α-OH sugar **9** and dihydroartemisinin in the presence $BF_3{\cdot}Et_2O$ as catalyst (Figure 2) gave β-glucuronide (tri-OAc ester) of β-dihydroartemisinin (**13**) with minor amounts of **11** and **12**.

(C) Isolation and Characterization of Glucuronide Tri-OAc Ester Derivatives of Dihydroartemisinin. The reactions were monitored by TLC with the aid of *p*-anisaldehyde spray reagent. A combination of TLC solvent systems were developed to help clearly identify, differentiate, and separate the isomeric glucuronide tri-OAc derivatives of dihydroartemisinin (**10**–**13**) from the other byproducts of the reaction. The glucuronide tri-OAc conjugates were characterized by ^{1}H-NMR (Table 1), ^{13}C-NMR (Table 2), and thermospray LC/MS. The compounds showed (100%) pseudomolecular ions ($M + NH_4^+ = m/z$ 618) by thermospray mass spectroscopy. The block of the ion source was operated at a temperature of 190 °C since the best sensitivity of arteether metabolites was observed[9] at a low block temperature.

Table 1. ^{1}H-NMR Chemical Shift Assignments of the Glucuronides of Dihydroartemisinin

	10		**11**	**12**		**13**	**15**		**17**	**18**
proton no.	$CDCl_3$	C_6D_6	($CDCl_3$)	$CDCl_3$	C_6D_6	($CDCl_3$)	$CDCl_3$	C_6D_6	(D_2O)	(D_2O)
11(1H)	2.36 m	2.64 m	2.61 m	~1.65	~1.45	2.63 m	3.25 m	3.53 m	2.35 m	2.53 m
12(1H)	4.78 d	4.90 d	4.86 d	5.14 d	5.32 d	4.98 d	9.80 d	10.01 d	4.74 d	5.07 d
	(9.5)	(9.2)	(3.4)	(3.1)	(3.5)	(3.5)	(1.6)	(1.0)	(9.5)	(3.3)
13(3H)	0.90 d	0.83 d	0.79 d	1.27 d	1.23 d	0.83 d	1.08 d	1.27 d	0.90 d	0.97 d
	(7.0)	(7.1)	(7.4)	(7.5)	(7.3)	(7.3)	(7.3)	(7.3)	(7.0)	(7.3)
14(3H)	0.95 d	0.69 d	0.94 d	0.96 d	0.70 d	0.94 d	0.94 d	0.57 d	0.95 d	0.93 d
	(5.6)	(6.0)	(5.7)	(6.4)	(5.9)	(6.3)	(6.2)	(6.0)	(5.6)	(6.3)
1′(1H)	~5.07	5.25 d	6.43 d	~5.49	5.75 d	4.78 d	5.08 d	5.17 d	4.57 d	4.53 d
		(7.2)	(4.0)		(3.6)	(7.9)		(7.8)	(7.6)	(7.8)
2′(1H)	~5.06	5.44 t	4.13 dd	5.05 dd	5.17 dd	5.04 t	5.03 t	5.51		~3.35
		(7.2, 8.6)	(4.0, 9.9)	(3.6, 10.2)	(3.6, 10.2)	(7.8, 8.5)	(8.5)			

Table 2. ^{13}C-NMR Chemical Shift Assignments of the Glucuronides of Dihydroartemisinin and Hydroxyarteethers

carbon no.	**10** ($CDCl_3$)	**11** ($CDCl_3$)	**12** ($CDCl_3$)	**13** ($CDCl_3$)	**15** ($CDCl_3$)	**18** (D_2O)	**19** ($CDCl_3$)	**21** ($CDCl_3$)	**22** ($CDCl_3$)	**26** (D_2O)
1(1H)	51.5	52.3	52.2	53.9	53.4	54.7	51.7	57.2	47.1	52.0
2	24.7 (2H)	24.8 (2H)	24.8 (2H)	25.9 (2H)	25.6[i] (2H)	26.8 (2H)	24.0 (2H)	80.2 (Hβ)	24.1 (2H)[q]	26.6 (2H)
3(2H)	36.3	36.3	36.4	37.8	35.8[j]	38.4	37.6	46.6	36.3	38.2
4(0H)	104.2	104.2	103.6	105.3	104.6	108.1	105.4	102.6	104.1	108.1
5(1H)	91.2	87.9	88.5	89.9	91.4	91.5	88.8	87.7	87.7	90.4
6(0H)	80.2	80.7	81.1	82.2	85.2	85.2	81.3	80.4	81.0	84.0
7(1H)	45.3	44.0	45.8	45.5	49.2	43.4	41.8	44.5	44.3[r]	43.8
8(2H)	22.2	24.1	31.4	25.3	25.5[i]	26.6	33.3	24.2	24.1[q]	34.3
9	34.2 (2H)	34.5 (2H)	34.6 (2H)	35.9 (2H)	35.6[j] (2H)	36.6 (2H)	86.7 (Hα)	35.7 (2H)	29.0 (2H)	86.7 (Hα)
10(1H)	37.4	37.7	37.6	38.5	38.0	39.5	43.7	36.8	42.5[r]	45.1
11(1H)	32.4	30.4	38.6	32.0	45.2	33.8	30.9	30.8	30.7	33.2
12(1H)	96.3	101.4	101.1	105.1	206.9	106.6	102.7	101.8	101.7	106.2
13(3H)	12.3	12.6	19.7	13.9	16.4	14.8	14.1	13.0	13.0	14.6
14	20.3 (3H)	20.3 (3H)	20.2 (3H)	21.6 (3H)	20.0 (3H)	22.3 (3H)	16.3 (3H)	21.7 (3H)	72.3 (2H)	17.1 (3H)
15(3H)	25.9	26.0	25.8	27.4	25.6	27.7	26.1	26.1	26.2	27.7
16(2H)							65.4	64.0	63.9	66.8
17(3H)							15.5	15.2	15.2	17.0
1′(1H)	96.0	88.2	93.5	101.1	94.3	105.8	103.6	100.7	100.8	104.2
2′(1H)	72.6	73.4	69.8	72.7	70.2	75.9	72.8	71.6	71.2	76.2
3′(1H)	72.0	70.5	69.4	73.5	72.1	78.3	73.6	69.1	72.0	74.6[u]
4′(1H)	70.9	70.3	69.6	70.6	69.6	74.4	70.6	72.6	69.4	78.4[u]
5′(1H)	69.5	69.7	69.1	73.6	72.7	79.0	73.5	71.9	72.6	78.7
2′-(3H)	20.8[a]	21.0[e]	20.7[c]	21.9[g]	20.6[k]		22.0[m]	20.8[o]	20.5[s]	
3′-(3H)	20.7[a]	20.8[e]	20.6[c]	21.9[g]	20.6[k]		22.0[m]	20.6[o]	20.6[s]	
4′-(3H)	20.5[a]	20.5[e]	20.5[c]	21.9[g]	20.4[k]		21.9[m]	20.5[o]	20.6[s]	
5′-OCH_3	52.8	53.0	52.9	54.0	52.8		54.2	52.8	52.9	
2′-C=O	169.7[b]	169.5[f]	169.7[d]	170.8[h]	169.3[l]		170.7[n]	169.4[p]	169.4[t]	
3′-C=O	169.5[b]	168.6[f]	168.5[d]	170.4[h]	168.9[l]		169.8[n]	169.1[p]	169.0[t]	
4′-C=O	167.5[b]	167.5[f]	168.0[d]	168.5[h]	167.0[l]		168.4[n]	167.0[p]	167.2[t]	
5′-C=O	170.1	170.2	170.1	171.4	170.2	178.5	171.6	170.2	170.2	178.5

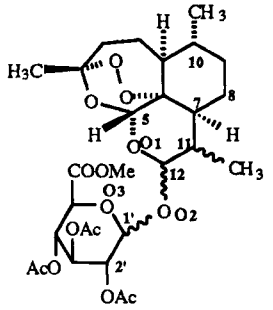
[a-u] Signals bearing the same superscript may have interchangeable assignments.

The stereochemistry of these products with respect to dihydroartemisinin and the sugar portions was determined by extensive analysis of the 300 MHz ^{1}H-NMR spectrum and the COSY 2D NMR of each compound. For the sugar portion of the molecule, the coupling constant of proton H-1′ is indicative of stereochemistry.[21] For an α-glucuronide H-1′ is a doublet with $J_{1',2'} = 4.00$ Hz, while in the case of a β-glucuronide H-1′ is a doublet with $J_{1',2'} = 7.80$ Hz. The key NMR feature that characterizes the dihydroartemisinin portion of the product is the coupling constant for the H-12 proton.[13,22] For β-arteether (similarly in β-dihydroartemisinin) H-12 is a doublet with $J_{11,12} = \sim 3.50$ Hz, whereas in the case of α-arteether (similarly in α-dihydroartemisinin) H-12 is a doublet with $J_{11,12} = \sim 9.25$ Hz. However, this simple stereochemistry rule could not be applied to the 11-epi series (because H-11 was now equatorial), thus additional 2D nuclear Overhauser studies and molecular modeling studies (as delineated below) were needed to determine the stereochemistry at C-12 and C-11.

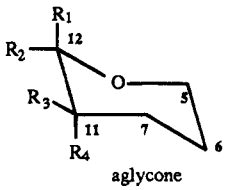
In the case of **10**, proton H-12 was a doublet and had a coupling constant of 9.5 Hz in $CDCl_3$ and 9.2 Hz in C_6D_6 as solvents indicating α-stereochemistry for dihydroartemisinin portion of the molecule. The ^{1}H-NMR spectra of **10** was acquired in deuterated benzene as solvent to clearly see the coupling for H-1′ which was obscured in the spectra acquired using deuterated chloroform as solvent. Proton H-1′ coupled to H-2′ with a coupling constant of 7.23 Hz, indicating β-stereochemistry for the sugar portion of the molecule. In the case of **11**, proton H-12 had a coupling constant of 3.40 Hz, indicating β-stereochemistry for the dihydroartemisinin portion of the molecule. Proton H-1′ coupled to H-2′ with a coupling constant of 4.00 Hz, indicating α-stereochemistry for the sugar portion of the molecule. In the case of **13**, proton H-12 had a coupling constant of 3.48 Hz, indicating β-stereochemistry for dihydroartemisinin, and H-1′ coupled to H-2′ with a coupling constant of 7.80 Hz, indicating β-stereochemistry for the sugar.

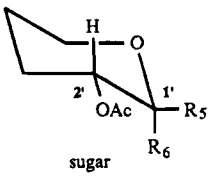
For **12**, $J_{11,12}$ was found to be 3.1 Hz which would suggest 12β,11β-stereochemistry, but the chemical shifts of H-11, H-13, C-8, C-11, and C-13 were more characteristic of the 11α-(epi) stereochemistry as reported for 11-epi-β-deoxyarteether.[19] For the 12α,11β-

Table 3. Observed and Calculated Coupling Constants of Stereochemical Importance for the Possible Diastereomers of the Glucuronide Tri-OAc Ester Derivatives of Dihydroartemisinin as Compared to Arteether and the Sugars



possible isomers of dihydroartemisinin glucuronide tri-OAc ester derivatives





Conformations of key groups for the various isomers

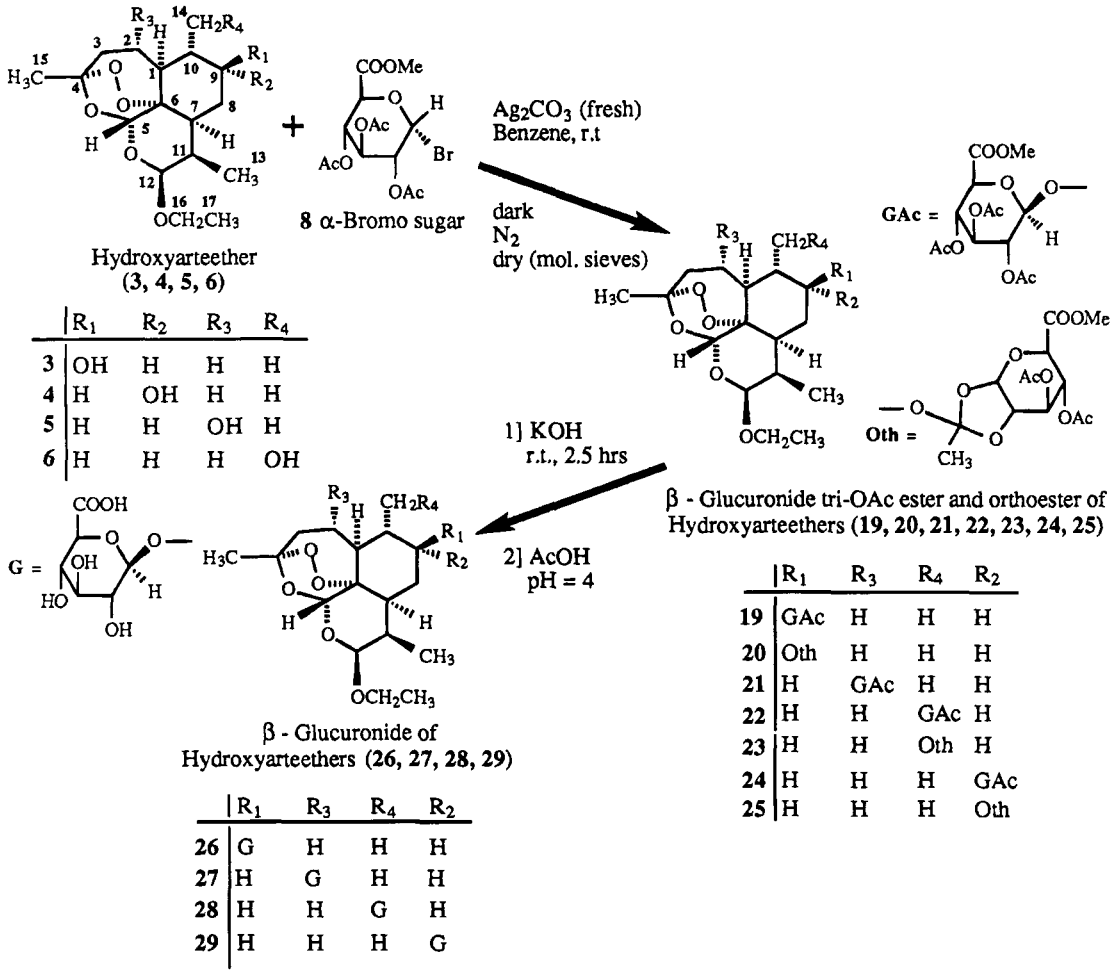
compound and stereochemistry	R_1 β	R_2 α	R_3 β	R_4 α (epi)	R_5 β	R_6 α	$J_{11,12}$ (Hz) obsd	$J_{11,12}$ (Hz) calcd[a]	$J_{1',2'}$ (Hz) obsd	$J_{1',2'}$ (Hz) calcd[a]
12β,11β (**1**)	OCH_2CH_3	H	CH_3	H			3.6	3.4		
12α,11β (α-arteether)	H	OCH_2CH_3	CH_3	H			9.0			
1'α (**9**)					H	OH			4.4	
1'β (**14**)					OAc	H			7.8	
12α,11β,1'β (**10**)	H	sugar	CH_3	H	agly	H	9.2	11.6	7.2	10.6
12β,11β,1'α (**11**)	sugar	H	CH_3	H	H	agly	3.4	1.9	4.0	1.0
12β,11α,1'α (**12**)	sugar	H	H	CH_3	H	agly	3.1	0.8	3.6	0.7
12β,11β,1'β (**13**)	sugar	H	CH_3	H	agly	H	3.5	2.0	7.8	10.8
12α,11β,1'α	H	sugar	CH_3	H	H	agly		11.5		1.0
12β,11α,1'β	sugar	H	H	CH_3	agly	H		0.8		10.8
12α,11α,1'β	H	sugar	H	CH_3	agly	H		2.2		10.7
12α,11α,1'α	H	sugar	H	CH_3	H	agly		2.3		1.4
11β,1'β (**15**)					agly	H	1.0		7.8	

[a] Using dihedral angles obtained from the energy minimized models of the compounds and Karplus-type equation.

stereochemistry, molecular modeling calculations gave an estimated $J_{11,12}$ value of 11.5 Hz (axial–axial coupling) (Table 3), which was clearly out of the range of the observed value (3.1 Hz). However, molecular modeling gave $J_{11,12}$ estimates of 2.0, 0.8, and 2.2 Hz for the remaining three possible combinations (12β,11β; 12β,-11α; 12α,11α, respectively) of the 11,12-stereochemistry. Given the above characteristic changes in the shifts and the molecular modeling estimates of $J_{11,12}$ values, it was clear that **12** had the 11α-configuration, but the configuration at C-12 remained uncertain. To resolve this uncertainty, the 2D NOESY spectrum of **12** was obtained and compared to the previously reported[22] 2D NOESY spectrum of β-arteether. While β-arteether showed strong cross-peaks between protons 12α,8β and 12α,5, the absence of these same cross-peaks in the 2D NOESY spectrum of **12** showed that it must have had a 12β,11α-configuration. Using the 2D NOESY spectrum of **12**, key proton to proton distances were measured and compared to the distances obtained by molecular modeling to give additional support for the proposed stereochemistry. The ^{1}H-NMR spectra of **12** was acquired in deuterated benzene as solvent to clearly see the coupling for 1' which was obscured in the spectra acquired using deuterated chloroform as solvent. Proton H-1' was a doublet with a coupling constant of 3.55 Hz, indicating α-stereochemistry for the sugar.

The downfield shift of the doublet ($J_{11,12}$ = 1.02 Hz, in C_6D_6 as solvent) for H-12 to 10.0 ppm and C-12 to 207.0 ppm in the case of **15** substantiated by the APT spectra indicated that the carbon C-12 was an aldehydic carbonyl. To add, proton H-11 showed cross-peaks in the COSY spectrum to H-12 and H-13. Proton H-1' was a doublet with a coupling constant of 7.80 Hz, indicating β-stereochemistry for the sugar.

(D) Hydrolysis of the Glucuronide Tri-OAc Derivatives of Dihydroartemisinin and Isolation and Characterization of the Glucuronides of Dihydroartemisinin. The ratio of water to methanol as solvents and the molar ratio of KOH to the glucuronide tri-OAc derivatives of dihydroartemisinin were optimized to ensure complete hydrolysis of **10** and **13** with minimal decomposition. The reaction was monitored by thermospray LC/MS ($M + NH_4^+ = m/z$ 618 for the glucuronide tri-OAc derivative **10** or **13**; $M + NH_4^+ = m/z$ 478 for the glucuronide **17** or **18**) for the disappearance of the peak corresponding to the glucuronide tri-OAc ester derivative and the appearance of one corresponding to the product. TLC was also used as a means of evaluating the progress of the hydrolysis, and the products of the reaction were visualized by means of *p*-anisaldehyde spray reagent. The presence of small amounts of water is required for the hydrolysis of the methyl ester functionality. No unreacted glucuronide tri-OAc derivative remained in the reaction mixture after 1 h (Figure 2). The use of too short a reaction time resulted in the formation of the glucuronide free acid methyl ester intermediate ($M + NH_4^+ = m/z$ 492), while longer reaction times or too much KOH resulted in decomposition (decomposition product $M + NH_4^+ = m/z$ 256) of the glucuronide target compound. The reaction was arrested with glacial acetic acid after 2.5 h, the solvent was removed under high vacuum at room temperature, and then the product was purified by reversed-phase chromatography. The glucuronide conjugates were characterized by ^{1}H-NMR in D_2O as



	R1	R2	R3	R4
3	OH	H	H	H
4	H	OH	H	H
5	H	H	OH	H
6	H	H	H	OH

	R1	R3	R4	R2
19	GAc	H	H	H
20	Oth	H	H	H
21	H	GAc	H	H
22	H	H	GAc	H
23	H	H	Oth	H
24	H	H	H	GAc
25	H	H	H	Oth

	R1	R3	R4	R2
26	G	H	H	H
27	H	G	H	H
28	H	H	G	H
29	H	H	H	G

Figure 3. Chemical synthesis of glucuronides of the hydroxyarteethers.

Table 4. Reaction Conditions for the Chemical Synthesis of the Glucuronide Tri-OAc Ester Derivatives of the Hydroxyarteethers

compd	hydroxyarteether [328.0]	α-Br sugar [397.2]	Ag_2CO_3 [275.7]	time (days)	yield, mp
19	**3**, 50 mg, 0.15 mmol (×1)	59.58 × 8 mg, 1.2 mmol (×8)	62.03 × 8 mg, 1.8 mmol (×12)	8	10.35%, 121 °C
21	**5**, 15 mg, 0.046 mmol (×1)	25.82 × 10 mg, 0.65 mmol (×14)	25.36 × 10 mg, 0.92 mmol (×20)	10	19.75%, 139 °C
22	**6**, 10 mg, 0.03 mmol (×1)	47.66 × 3 mg, 0.36 mmol (×12)	49.63 × 3 mg, 0.54 mmol (×18)	3	36.23%, 108 °C
24	**4**, 1 mg, 0.003 mmol (×1)	1.28 × 14 mg, 0.045 mmol (×15)	1.18 × 14 mg, 0.06 mmol (×20)	14	15.67%, 130 °C

solvent (Table 1) for the loss of the OAc and methyl ester singlets and making certain that the stereochemistry of H-12 for the dihydroartemisinin portion of the molecule and H-1′ for the sugar portion of the molecule remained the same as in their corresponding glucuronide tri-OAc ester derivative.

(E) Synthesis, Isolation, and Characterization of the Glucuronide Tri-OAc Ester Derivatives of the Hydroxyarteethers. The glucuronide tri-OAc ester derivative of 9β-hydroxyarteether (**3**) was synthesized using α-bromo sugar **8** and Ag_2CO_3 as a catalyst (Figure 3). The chemical synthesis was monitored by TLC (10:90 EtOAc/CH_2Cl_2), and the products of the reaction were visualized by means of *p*-anisaldehyde spray reagent. In comparison to the synthesis of the glucuronides of dihydroartemisinin, the reaction seemed to be very slow but showed formation of two products. Since both of the products showed molecular ions ($M + NH_4^+ = m/z$ 662) by thermospray mass spectroscopy, it was presumed that one of the products was the desired β-glucuronide tri-OAc ester derivative **19** and the other either the α-glucuronide derivative or the ortho ester byproduct **20** (all have the same mass). Using the molar ratios of sugar:Ag_2CO_3:aglycone as 8:12:1, respectively (sugar and Ag_2CO_3 were added in eight equal portions; Table 4), at room temperature and benzene as solvent gave the best yields of β-glucuronide (tri-OAc ester) of 9β-hydroxyarteether (**19**). In using this procedure the formation of the α-glucuronide byproduct (characterized by ^{1}H-NMR, doublet for H-1′ with $J_{1',2'} = 4.0$ Hz) was not detected, but a small amount of the ortho ester byproduct **20** (^{1}H-NMR showed a doublet for H-1′ at δ 5.8 with $J_{1',2'} = 5.8$ Hz and a 3H singlet at δ 1.70) was isolated from the reaction (Figure 3). Similarly β-glucuronide (tri-OAc ester) of 2α-hydroxyarteether (**21**), β-glucuronide (tri-OAc ester) of 14-hydroxyarteether (**22**), and β-glucuronide (tri-OAc ester) of 9α-hydroxyarteether (**24**) were synthesized using the Ag_2CO_3 procedure using different molar ratios (Table 4) of the sugar:Ag_2CO_3:aglycone (Figure 3). The stereochemistry of the product with respect to the hydroxyarteether and the sugar portions were determined by analysis of the 300 MHz proton NMR spectrum and COSY 2D NMR of the compound. The key NMR features that characterizes the aglycone portion of the glucuronide tri-OAc ester derivatives of the hydroxyarteethers are the

Table 5. ^{1}H-NMR Chemical Shift Assignments of the Glucuronides of Hydroxyarteethers

proton no.	**19** ($CDCl_3$)	**21** ($CDCl_3$)	**22** ($CDCl_3$)	**24** ($CDCl_3$)	**26** (D_2O)	**27** (D_2O)	**28** (D_2O)	**29** (D_2O)
2β(1H)	1.60 m	3.89 m			1.61m	3.89 m		
9α(1H)	3.00 ddd				3.00 ddd			
	(3.8, 10.1, 10.1)				(4.5,8.2,8.2)			
9β(1H)				~3.74				~3.80
11(1H)	2.58 m	2.61 m	2.62 m	2.63 m	2.58 m	2.51 m	2.62 m	2.52 m
12(1H)	4.79 d	4.78 d	4.79 d	4.81 d	4.79 d	4.78 d	4.79 d	4.80 d
	(3.3)	(3.3)	(3.2)	(2.7)	(3.4)	(3.3)	(3.2)	(3.2)
13(3H)	0.89 d	0.89 d	0.90 d	0.91 d	0.89 d	0.92 d	0.90 d	0.86 d
	(7.1)	(7.3)	(7.4)	(7.3)	(7.1)	(7.7)	(7.2)	(7.3)
14	0.97 (3H) d	0.95 (3H) d	3.86 (Hα) m	1.03 (3H) d	0.97 (3H) d	1.06 (3H) d	3.3 - 4.1	0.96 (3H) d
	(6.1)	(5.8)	3.46 (Hβ) m	(6.7)	(6.1)	(6.2)		(6.7)
1′(1H)	4.61 d	4.74 d	4.52 d	4.54 d	4.61 d	4.67 d	4.39 d	4.48 d
	(7.9)	(7.7)	(7.6)	(7.6)	(7.9)	(7.7)	(8.1)	(7.8)

coupling patterns for the H-9α proton in **19**, H-9β proton in **24**, H-2β proton in **21**, and H-14 proton in **22**, all of which are listed in Table 5. In **19**, **21**, **22**, and **24** H-1′ of the sugar was a doublet with coupling constant $J_{1',2'} \sim 7.8$ Hz, indicating β-stereochemistry for the glucuronyl portion of the product. The ^{1}H- and ^{13}C-NMR data of the glucuronide (tri-OAC esters) of the hydroxyarteethers are listed in Tables 5 and 2, respectively.

(F) Hydrolysis of the Glucuronide Tri-OAc Derivatives of the Hydroxyarteethers and Isolation and Characterization of the Glucuronides of the Hydroxyarteethers. Conditions similar to those used for the conversion of glucuronides (tri-OAc ester) of dihydroartemisinin to their free acid forms were used for the conversion of all the glucuronides (tri-OAc ester) of the hydroxyarteethers **19**, **21**, **22**, and **24** to their free acid forms (**26**–**29**) (Figure 3). The hydrolysis reaction was monitored by thermospray LC/MS and TLC (20:80 EtOAc/CH_2Cl_2 and 74% EtOAc + 15% MeOH + 10% H_2O + 1% AcOH) for the disappearance of the glucuronide tri-OAc ester derivatives of the hydroxyarteethers **19**, **21**, **22**, and **24** and the appearance of the product β-glucuronides of the hydroxyarteethers (**26**–**29**). The reaction was completed in 2.5 h. The decomposition products and the inorganics in the reaction mixture were removed by means of a small scale C_{18} reversed-phase column chromatography. The glucuronide conjugates were characterized by ^{1}H-NMR in D_2O as solvent (Table 5) for the loss of the OAc and methyl ester singlets and making sure that the coupling constants for H-12 of the arteether portion of the molecule and H-1′ of the sugar portion of the molecule remained almost the same as in their corresponding glucuronide tri-OAc ester derivative.

Molecular Modeling Studies of the Glucuronide Tri-OAc Derivatives of Dihydroartemisinin for Stereochemical Identification. Molecular mechanics energy minimization molecular modeling was used to arrive at the structures of the possible isomers of glucuronide tri-OAc derivatives of dihydroartemisinin (differing at the three asymmetric carbons 12, 11, and 1′) (Table 3), and the molecular modeling results were compared to the NMR data. An initial representation of the structures of all possible isomers was generated using Chem 3D Plus (MM2 force field) molecular mechanics energy minimizations of the whole molecule with no distance constraints. The molecules were then subjected to molecular dynamics calculations (heating to 500 K and then cooling to 0 K) and further minimized to obtain the global minimum. Key dihedral angles of the possible isomers were calculated from which their corresponding coupling constants were obtained using a Karplus-type equation[23] by means of a semiempirical approach (eq 1) that takes into account the heteroatom substitution on the adjacent carbon atoms.[24,25]

$$J = A + B\cos\theta + C\,2\theta + \cos\theta[(\Delta S_1 + \Delta S_4)\cos(\theta - 120) + (\Delta S_2 + \Delta S_3)\cos(\theta + 120)] \quad (1)$$

The basic ring systems of both dihydroartemisinin and the sugar have a number of vicinal protons, and of these protons the dihedral angles of stereochemical importance are H-11 to H-12 and H-1′ to H-2′ (Table 3). The coupling constants were obtained from the calculated dihedral angles using the Karplus-type equation. The observed coupling constants and dihedral angles obtained from the NMR data were then compared to the calculated values obtained from the molecular mechanics energy minimization (Table 3) to arrive at a final structure reflecting the actual structure of all the possible isomers of glucuronide tri-OAc derivatives of dihydroartemisinin in solution. Some key H–H distances were calculated which helped determine the solution conformation and 3D spatial arrangement of the various portions of the molecule. For **12**, the volume of the 2D NOESY cross-peaks were used to estimate the H–H distances; then, these experimental distances were compared to the values obtained by molecular modeling.

Table 6. HPLC Retention Indices and log *P* Values for Glucuronides and Their Aglycones

compd	retention index (*I*)	log *P*	shift in I^a
26	464	0.61	−264
29	529	0.93	−242
28	551	1.05	−207
17	560	1.08	−231
27	688	1.73	−193
18	701	1.80	−130
3	728	1.93	
6	758	2.08	
4	771	2.14	
2	831	2.45	
5	881	2.70	
1	1120	3.89	

[a] Shift between the retention index of the glucuronide and its aglycone.

Determination of log *P* Values. The retention index for each compound (**1**–**6**, **17**, **18**, and **26**–**29**) was determined with a C-18 HPLC system, and log *P* of each compound (Table 6) was estimated from its corresponding retention index.[26,27] The log *P* values of the glucuronides (0.61–1.80) were considerably lower than that of arteether, but well within the 1.0–2.0 range that was

Table 7. *In Vitro* Antimalarial Activity of Arteether and Its Glucuronides Compared to Their log *P* Values

compound	IC_{50} (ng/mL)	log IC_{50}	log P^a
9β-hydroxyarteetherglucuronide (**26**)	89.3	1.951	0.61
9α-hydroxyarteetherglucuronide (**29**)	6560.0	3.817	0.93
14-hydroxyarteetherglucuronide (**28**)	6000.0	3.778	1.05
α-dihydroartemisininglucuronide (**17**)	6000.0	3.778	1.08
2α-hydroxyarteetherglucuronide (**27**)	605.0	2.781	1.73
β-dihydroartemisininglucuronide (**18**)	255.0	2.406	1.80
dihydroartemisinin (**2**)	10.7	1.030	2.45
arteether (**1**)	1.3	0.124	3.89

[a] Using the HPLC retention index method.

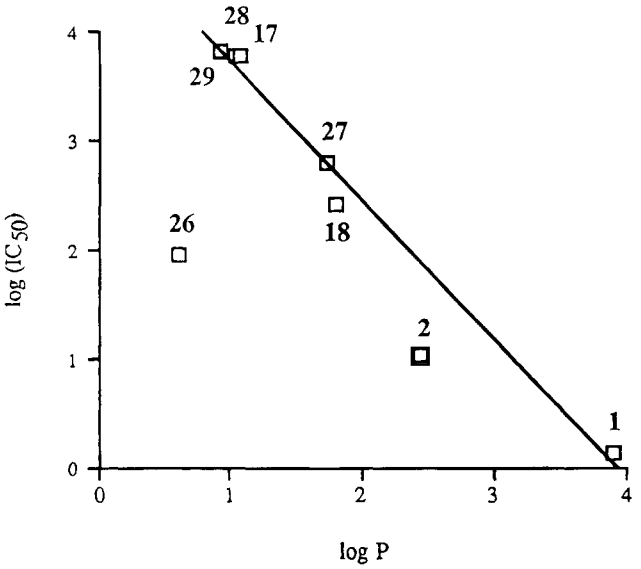


Figure 4. Activity to log *P* relationship of arteether and its glucuronides. The numbers adjacent to the points on the graph are the corressponding compound numbers.

needed for good biological activity. The average shift between the retention index of the glucuronide and it's aglycone was found to be in agreement with the value of −247 that had been reported previously[27] for a variety of glucuronides.

Antimalarial Activity. The *in vitro* antimalarial activity of arteether and several of the reference standards was determined using *P. falciparum*, strain FCR-3, as a model (Table 7). The glucuronide of β-dihydroartemisinin (**18**) was around 20 times less active than its aglycone **2**, while the α-isomer **17** was found to be almost inactive. This observation was consistent with the previous report[28] that has shown that 12β-ethers were more active than the 12α-isomers. Among the 9-hydroxyarteether glucuronides, the glucuronide of 9β-hydroxyarteether (**26**) was the most potent of all the glucuronide conjugates and around 1000 times less active than its aglycone **3**,[9] while the other hydroxyarteether glucuronides had very low activity. In general the glucuronides follow a linear relationship between log *P* and activity (Figure 4) similar to that of their parents with the exception of **26**.

Discussion

The hydroxylated metabolites **1**−**6** of arteether (**1**) were obtained either chemically or by microbial biotransformation. Their glucuronide conjugates **17** and **18** were synthesized using two general methods to form the glucuronide tri-OAc derivatives. The two-step Koenigs−Knorr chemical synthetic procedure using α-bromo sugar and Ag_2CO_3 as catalyst gave primarily β-glucuronide of α-dihydroartemisinin by an S_N2 mechanism (Walden's, inversion of configuration at 1′ of the sugar in the product), while the other procedure using α-hydroxy sugar and $BF_3{\cdot}Et_2O$ as catalyst gave primarily β-glucuronide of β-dihydroartemisinin by an S_N1 mechanism. The Koenigs−Knorr procedure was used to form the glucuronide tri-OAc derivatives **26**−**29** of the hydroxylated metabolites. Conversion of the glucuronide tri-OAc derivatives to their free acid forms was done using aqueous KOH. Complete spectral characterization of the glucuronide conjugates was done as their tri-OAc derivatives.

Molecular modeling, extended Karplus coupling analysis, and quantitative 2-dimensional nuclear Overhouser spectra were used to determine the stereochemistry and conformation of the four isomeric glucuronides of dihydroartemisinin. As expected from earlier studies,[22] the calculated H−H distances for protons 5−8β, 5−10, 11−7, and 11−12 were about the same for all the isomers (refer to table in the supplementary material). In the case of 12α- (H-12 is β and axial) isomers, one would expect to see NOE cross-peaks (cross-peaks usually observed for 2.00−3.00 Å distances) between protons 5−12β (~2.4 Å) and 12β−8β (~2.3 Å) and not in case of the 12β- (H-12 is α and axial) isomers (5−12α, ~3.7 Å, and 12α−8β, ~4.0 Å). These distances were independent of the configuration at H-11. Thus the presence or absence of these NOE cross-peaks confirms the stereochemistry at C-12 as α or β, respectively. The stereochemistry at C-11 was assigned epi (α) by the presence of NOE cross-peaks between protons 11−8α (2.4 Å) and the lack of a cross-peak between protons 13−8α (4.0 Å).

The molecular modeling studies were also used to estimate the values of $J_{11,12}$ and $J_{1',2'}$ for the four isomeric dihydroartemisinin glucuronides as an additional means of determining their stereochemistry. The precision of *J*-value estimates are typically ±2.0 Hz,[24,25] and for this series there was a satisfactory agreement between the experimental and estimated $J_{11,12}$ values: **10**, 9.2 Hz (estimated 11.6); **11**, 3.4 Hz (estimated 1.9); **12**, 3.0 Hz (estimated 0.8); **13**, 3.5 Hz (estimated 2.0) (Table 3). The observed coupling constant of ~3.00 Hz for $J_{11,12}$ in the case of **12** agreed well with that calculated for the 12α,1′α,11α-isomer, but still **12** was assigned 12β,1′α,11α-isomer on the basis of the NOE data. The lack of NOE cross-peaks between H-12 and H-8β or H-5 agreed well with the calculated distances between the protons (>3.0 Å) in the case of the 12β,1′α,11*E*-isomer, where as in the case of the 12α,1′α,11*E*-isomer one should have observed the NOE cross-peaks (2.0−3.0 Å) (Table 3). This confirmed the stereochemistry of the dihydroartemisinin portion of **12** as 11-epi-β-dihydroartemisinin. Similarly, the observed coupling constant for $J_{1',2'}$ was in agreement with the calculated value.

A very good correlation was found between the log *P* of the glucuronide metabolites and their *in vitro* antimalarial activity, with the exception of **26** (Figure 4). As might be expected, as the lipophilicity of the compound increased, there was an increase in activity. While the glucuronide of the typical xenobiotic would have far too low of a lipophilicity to show activity, several of the arteether glucuronides retained sufficient

lipophilicity to confer antimalarial activity. A notable exception to the log P–antimalarial activity trend was β-glucuronide of 9β-hydroxyarteether (**26**), which was the least lipophilic yet the most active of the glucuronides. The exception indicates a possible greater influence of the stereochemical or conformational factors of these compounds on their activity. It was also noteworthy that β-glucuronide of 9β-hydroxyarteether was almost 2 orders of magnitude more active than its corresponding 9α-isomer **29**. This finding was consistent with the earlier report where the antimalarial activity of 9β-hydroxyarteether was found to be more than 2 orders magnitude greater than its corresponding 9α-isomer.[9] Of the compounds prepared for the present study, clearly 9β-hydroxyarteether-β-glucuronide warrants further consideration as a therapeutic agent for intravenous use and with regard to its contribution to the activity observed for arteether *in vivo*.

Experimental Section

General. Thermospray LC/MS spectra were obtained using a Vestec Model 201 mass spectrometer with a Technivent data system and thermospray interface. The standard pulse sequences of the Varian VXR-300 spectrometer were employed for ^{1}H-NMR, ^{13}C-NMR, APT, DEPTGL, HETCOR, COSY, and NOESY spectra (operating at 300 MHz for proton and 75 MHz for carbon). The spectra were recorded in either deuterated chloroform ($CDCl_3$) or deuterated benzene (C_6D_6) (internal standard as tetramethylsilane in both cases) or deuterated water (D_2O) (internal standard as sodium 2,2-dimethyl-2-silapentane-5-sulfonate) as solvent. Thin-layer chromatographic (TLC) analyses were carried out on precoated aluminum backed (Art. 5554, DC-Alufoilen Kieselgel) silica gel 60 F_{254} plates (0.2 mm, E. Merck). TLC plates were developed in a suitable solvent and visualized with *p*-anisaldehyde-sulfuric acid spray reagent followed by heat. The *p*-anisaldehyde–sulfuric acid spray reagent (200 mL) was prepared as follows: *p*-anisaldehyde (1 mL), glacial acetic acid (20 mL), methanol (169 mL), and concentrated sulfuric acid (10 mL) added in that order. Column chromatography was conducted with the aid of standard flash columns using silica gel 60 Art. 9385 (40–60 μm, E. Merck). Acetobromo-α-D-glucuronic acid methyl ester (α-Br sugar, cat. no. A 8292) (**8**) and methyl 1,2,3,4-tetra-*O*-acetyl-β-D-glucuronate (β-OAc sugar, cat. no. M 5890) (**14**) were obtained from Sigma Chemical Co. Elemental analyses of the target compounds (glucuronide conjugates) referred to in the manuscript were not obtained because of very small quantities of most of these compounds, the majority of which was used in the analytical methods development using the LC/MS.

Chemistry. Dihydroartemisinin (10). The procedure of Brossi et al.[12] was used for the synthesis of dihydroartemisinin.

Arteether (21). Arteether was obtained from Dr. Charles D. Hufford, Department of Pharmacognosy, University of Mississippi, which was in turn obtained from Sapec S. A. Fine Chemicals, Lugano, Switzerland.

Microbial Metabolism of Arteether by *C. elegans* and *S. lavendulae*. The two-stage fermentation procedure using *C. elegans* (ATCC 9245)[7] yielded 400 mg (41.7% yield) of 9β-hydroxyarteether (**3**) (R_f 0.65, mp 125 °C) and using *S. lavendulae* (L 105)[7] yielded 30 mg (10.7% yield) of 9α-hydroxyarteether (**4**) (R_f 0.56, mp 133 °C), 90 mg (32.1% yield) of 2α-hydroxyarteether (**5**) (R_f 0.47, liquid), and 30 mg (10.7% yield) of 14-hydroxyarteether (**6**) (R_f 0.36, mp 111 °C). Their authenticity was confirmed by TLC (60% Et_2O in hexane) and ^{1}H- and ^{13}C-NMR data[7] as well as by thermospray LC/MS which indicated a (100%) molecular ion, M + NH_4^+ = 346.

β-Glucuronide (Tri-OAc ester) of α-Dihydroartemisinin (10) (Figure 2). Freshly prepared[18] silver carbonate Ag_2CO_3 [275.7] (1295 × 3 mg, i.e., added three times, each portion at 24 h time intervals, 14.08 mmol) and 1α-bromo-2,3,4-triacetyl-α-D-glucopyranuronate methyl ester (**8**) [397.2] (α-Br sugar, 1165 × 3 mg, 8.8 mmol) were added to a solution of dihydroartemisinin [284.0] (**2**) (500 mg, 1.76 mmol) in dry benzene (75 mL) along with molecular sieves (1 g). The suspension was stirred at room temperature in the dark (protected from light using aluminum foil) under N_2 for 4 days. The reaction was monitored by TLC (10% EtOAc in CH_2Cl_2) with the aid of *p*-anisaldehyde spray reagent and heat at intervals of 24 h for the disappearance of dihydroartemisinin (R_f 0.22) while the α-Br sugar (R_f 0.75) underwent decomposition. The reaction mixture was filtered (cintered glass funnel) under suction to remove the Ag salts and the residue washed with excess EtOAc. The combined filtrate was dried over anhydrous Na_2SO_4 and the solvent evaporated on a roto-vap under vacuum (water bath at 40 °C) to give 3.9 g of crude product. This was then flash chromatographed on a silica gel column using 10% EtOAC in CH_2Cl_2 to yield 50 mg of a solid, the major byproduct anhydrodihydroartemisinin (R_f 0.90); 150 mg of impure product (R_f 0.54, (thermospray LC/MS indicated products of mol wt 376 and 600, ^{1}H-NMR also showed mixture of products); and 10 mg of an aldehydic byproduct (**15**) ($C_{28}H_{40}O_{14}$, mol wt 600, thermospray LC/MS indicated (100%) molecular ion M + NH_4^+ = *m/z* 618, R_f 0.48), ^{1}H-NMR (Table 1), ^{13}C-NMR (Table 2).

The impure product was then again flash chromatographed on a silica gel column using 10% EtOAC in benzene to yield 48 mg of an impure product (R_f 0.35 in 25% EtOAC in benzene, thermospray LC/MS indicated a single product of mol wt 600, devoid of the sugar impurity (R_f 0.31) of mol wt 376, i.e., M + NH_4^+ = *m/z* 394, but ^{1}H-NMR showed a mixture of products). This was then rechromatographed on a silica gel column using 60% Et_2O in hexane to give 10 mg of a solid, α-glucuronide (tri-OAc ester) of 11-epi-β-dihydroartemisinin (**11**) ($C_{28}H_{40}O_{14}$, mol wt 600, thermospray LC/MS indicated (100%) molecular ion, M + NH_4^+ = *m/z* 618, R_f 0.42), ^{1}H-NMR (Table 1), ^{13}C-NMR (Table 2); 3 mg of an oil, α-glucuronide (tri-OAc ester) of β-dihydroartemisinin (**12**) ($C_{28}H_{40}O_{14}$, mol wt 600, thermospray LC/MS indicated (100%) molecular ion M + NH_4^+ = *m/z* 618, R_f 0.37), ^{1}H-NMR (Table 1), ^{13}C-NMR (Table 2); and 25 mg of β-glucuronide (tri-OAc ester) of α-dihydroartemisinin (**10**) (0.047 mmol, 2.65% yield, $C_{28}H_{40}O_{14}$, mp 160 °C, mol wt 600, thermospray LC/MS indicated (100%) molecular ion M + NH_4^+ = *m/z* 618, R_f 0.32), ^{1}H-NMR (Table 1), ^{13}C-NMR (Table 2).

1α-Hydroxy-2,3,4-triacetyl-α-D-glucopyranuronate Methyl Ester (α-OH sugar) (9). Using a reported procedure[29] α-OH sugar **9** was obtained from α-Br sugar **8** as a mixture of α/β-OH sugar in the ratio 4:1.

β-Glucuronide (Tri-OAc ester) of β-Dihydroartemisinin (13) (Figure 2). To a solution of dihydroartemisinin (**2**) (250 mg, 0.88 mmol) and 1α-hydroxy-2,3,4-triacetyl-α-D-glucopyranuronate methyl ester (**9**) (α-OH sugar, 1.76 g, 5.28 mmol) in dry benzene (25 mL) along with molecular sieves (1 g) was rapidly added $BF_3{\cdot}Et_2O$ [141.9] (65.7 mg, 1.154 g/mL, 57 μL, 0.46 mmol), and the mixture was stirred at room temperature under N_2 for 24 h. The reaction was monitored by TLC (20% EtOAc in CH_2Cl_2) with the aid of *p*-anisaldehyde spray reagent and heat at intervals of 4 h for the disappearance of dihydroartemisinin (R_f 0.38). The benzene layer was washed (in a separatory funnel) with saturated aqueous NaOAc solution (10 mL) and water (10 mL) and dried over anhydrous Na_2SO_4 (2.5 g) and the solvent evaporated to give 1.7 g of crude product. This was then flash chromatographed on a silica gel column using 10% EtOAC in CH_2Cl_2 to yield byproducts β,α-bisdihydroartemisinin, artemether, and anhydrodihydroartemisinin; an impure mixture of products (R_f 0.80 in 20% EtOAC in CH_2Cl_2, thermospray LC/MS indicated products of mol wt 376 and 600, TLC using 60% Et_2O in hexane indicated the presence of α-glucuronide (tri-OAc ester) of 11-epi-β-dihydroartemisinin (**11**) and α-glucuronide (tri-OAc ester) of β-dihydroartemisinin (**12**)); and 90 mg of β-glucuronide (tri-OAc ester) of β-dihydroartemisinin (**13**) (0.15 mmol, 17% yield, $C_{28}H_{40}O_{14}$, mp 151 °C, mol wt 600, thermospray LC/MS indicated (30%) molecular ion M + NH_4^+ = *m/z* 618, R_f 0.63 in 20% EtOAC in CH_2Cl_2), ^{1}H-NMR (Table 1), ^{13}C-NMR (Table 2).

β-Glucuronide of α-Dihydroartemisinin (17) (Figure 2). To a solution of β-glucuronide (tri-OAc ester) of α-dihydroartemisinin (**10**) [600] (5 mg, 0.0083 mmol) in 4.0 mL of MeOH and 1.0 mL of H_2O was slowly added 5 N KOH (1.65 μL, 0.0083 mmol), and the mixture was stirred for 2.5 h. The reaction was monitored by TLC with the aid of *p*-anisaldehyde spray reagent and heat at intervals of 0.5 and 1.5 h for the disappearance of **10** (R_f 0.54 in 10% EtOAc in CH_2Cl_2, R_f 0.98 in 74% EtOAc + 15% MeOH + 10% H_2O + 1% AcOH). The formation of the glucuronide free acid methyl ester intermediate (R_f 0.10 in 10% EtOAc in CH_2Cl_2, R_f 0.60 in 74% EtOAc + 15% MeOH + 10% H_2O + 1% AcOH) decreased with time (thermospray LC/MS indicated a molecular ion, M + NH_4^+ = *m/z* 492). The reaction was completed in 2.5 h at which stage 3.3 μL of 5 N glacial AcOH was added to neutralize the excess KOH (if any) and make the pH acidic. The reaction mixture after being evaporated to dryness under vacuum was dissolved in water, loaded on to a C_{18} reversed phase column, and purified by reversed-phase column chromatography using 45% MeOH in H_2O. The purity of the beginning and end column fractions containing the product (white amorphous material) was checked by TLC (74% EtOAc + 15% MeOH + 10% H_2O + 1% AcOH). Pooling the column fractions containing the product and evaporating them to dryness on a Savant instrument yielded 3.1 mg of β-glucuronide of α-dihydroartemisinin (**17**): 0.0068 mmol, 78.5% yield; $C_{21}H_{32}O_{11}$, mol wt 460; mp 165 °C; (thermospray LC/MS indicated a (100%) molecular ion, M + NH_4^+ = *m/z* 478; R_f 0.23 in 74% EtOAc + 15% MeOH + 10% H_2O + 1% AcOH; ^{1}H-NMR, Table 1.

β-Glucuronide β-Dihydroartemisinin (18) (Figure 2). Similarly, hydrolysis of **13** (50 mg, 0.083 mmol) yielded 30 mg of β-glucuronide of β-dihydroartemisinin (**18**): 0.065 mmol, 78.25% yield; $C_{21}H_{32}O_{11}$, mol wt 460; mp 162 °C; thermospray LC/MS indicated a very weak (5%) molecular ion, M + NH_4^+ = *m/z* 478 and strong fragment of mass *m/z* 256; R_f 0.22 in 74% EtOAc + 15% MeOH + 10% H_2O + 1% AcOH; ^{1}H-NMR, Table 1; ^{13}C-NMR, Table 2.

β-Glucuronide (Tri-OAc ester) of the Hydroxyarteethers 19, 21, 22, and 24 (Figure 3). Ag_2CO_3 [275.7] and 1α-bromo-2,3,4-triacetyl-α-D-glucopyranuronate methyl ester (**8**) [397.2] (α-Br sugar) were added to each of the hydroxyarteethers (**3**–**6**) [328.0] in dry benzene (15 mL) along with molecular sieves (1 g) (Table 4). The suspension was stirred at room temperature in the dark (protected from light using aluminum foil) under N_2. The reaction was monitored by TLC (10% EtOAc in CH_2Cl_2) with the aid of *p*-anisaldehyde spray reagent and heat at intervals of 24 h for the disappearance of **3** (R_f 0.71), **4** (R_f 0.60), **5** (R_f 0.55), and **6** (R_f 0.44) while the α-Br sugar (R_f 0.75) underwent decomposition. The reaction mixture was filtered (cintered glass funnel) under suction to remove the Ag salts and the residue washed with excess EtOAc. The combined filtrate was dried over anhydrous Na_2SO_4 and the solvent evaporated on a Roto-vap under vacuum (water bath at 40 °C) to give the crude product. This was then flash chromatographed on a silica gel column using 10% EtOAC in CH_2Cl_2 to yield a partially pure product which was rechromatographed using 10% EtOAC in benzene. TLC (25% EtOAC in benzene) was used to pool the column fractions having the glucuronides (tri-OAc ester) of the hydroxyarteethers (**19** (R_f 0.43), **21** (R_f 0.40), **22** (R_f 0.35), **24** (R_f 0.42)): $C_{30}H_{44}O_{15}$, mol wt 644; thermospray LC/MS indicated (100%) molecular ion M + NH_4^+ = *m/z* 662; ^{1}H-NMR, Table 5; ^{13}C-NMR, Table 2. Small amounts of the ortho ester byproducts **20**, **23**, and **25** were formed during the synthesis of **19**, **22**, and **24**, respectively.

β-glucuronide of the Hydroxyarteethers 26–29 (Figure 3). To a solution of glucuronides (tri-OAc ester) of the hydroxyarteethers **19**, **21**, **22**, and **24** in 20% water in MeOH was slowly added 5 N KOH (1:1 molar ratio), and the mixture was stirred for 2.5 h. The reaction was monitored by TLC (in 10% EtOAC in CH_2Cl_2) with the aid of p-anisaldehyde spray reagent and heat at intervals of 0.5 and at 1.5 for the disappearance of **19**, **21**, **22**, and **24**. The reaction seemed to go to completion in 2.5 h, at which stage double the molar amounts of 5 N glacial AcOH was added to neutralize the excess KOH (if any) and bring the pH on the acidic side. The reaction mixture after being evaporated to dryness under vacuum was dissolved in water and loaded onto a C_{18} reversed-phase column and purified by reversed-phase column chromatography using 45% MeOH in H_2O. The purity of the beginning and end column fractions containing the product (white amorphous material) was checked by TLC (74% EtOAc + 15% MeOH + 10% H_2O + 1% AcOH). Pooling the column fractions containing the product and evaporating them to dryness under vacuum yielded the glucuronides of the hydroxyarteethers (**26** (R_f 0.35), **27** (R_f 0.28), **28** (R_f 0.23), **29** (R_f 0.33)) in approximately 77% yields: $C_{23}H_{36}O_{12}$, mol wt 504; thermospray LC/MS indicated molecular ion M + NH_4^+ = *m/z* 522; ^{1}H-NMR, Table 5; ^{13}C-NMR, Table 2.

Molecular Modeling and NMR Studies of the Glucuronide Tri-OAc Derivatives of Dihydroartemisinin. Molecular modeling was conducted using the Chem 3D Plus (version 3.0, Cambridge Scientific Computing) program. The dihydroartemisinin backbone was obtained from the molecular modeling construct of arteether[22] which was built with the aid of NOE data and X-ray data of artemether.[30] To begin, 1′-hydroxy-2,3,4-triacetyl-α-D-glucopyranuronate methyl ester (α-OH sugar) was built and minimized using the MM2 force field. Then dihydroartemisinin and the sugar were put to together, and the glucuronide structure was minimized holding the atoms of both the ring systems fixed. Altering the stereochemistry at C-12 and C-11 of dihydroartemisinin and C-1′ of the sugar resulted in eight possible isomers of the glucuronide tri-OAc derivatives of dihydroartemisinin, all of which were minimized. The MM2 parameters in the *CSC Chem3D Plus* program were used. The missing parameters for the C_4–O–O–C_6 bond were derived using Schnur's[31] systematic approach, whereby '81' (alkoxide oxygen) was used as the conversion for MM2 atom type '6' (one of the peroxide oxygens). Molecular dynamics computation (heating to 500 K, cooling back to 0 K, and then subjecting the molecule to energy minimizations) was used to come up with the minimum energy conformations. It consisted of a series of steps which occur at fixed intervals of about 2.0 fs (fs, 1.0 × 10^{-15} s). The Beeman algorithm for integrating the equations of motion with improved coefficients was used to compute new positions and velocities of each atom at every step.

$$x_i = x_i + v_i\Delta t + [(5a_i - a_i^{old})(\Delta t)^2]/8 \qquad (2)$$

Each atom (*i*) is moved according to formula 2 (along the *x* coordinates) with a velocity (v_i) and acceleration (a_i) along its x_i, y_i, and z_i Cartesian coordinates, where a_i^{old} is the corresponding acceleration in the previous step and Δt is the time between the current and last steps. The potential energy and its derivatives are then computed with respect to the new Cartesian coordinates.

The observed NOE distances in case of α-glucuronide (tri-OAc ester) of 11-epi-β-dihydroartemisinin (**11**) were measured using the geminal 3α–3β NOE distance as reference. The observed and calculated coupling constants calculated by eq 1 ($J_{11,12}$ and $J_{1',2'}$) as well as the dihedral angles and various proton-proton distances of the eight possible glucuronide tri-OAc ester derivatives of dihydroartemisinin were compared.

Determination of log *P* Values. The log *P* values were determined using an HPLC method.[26,27] The retention times were obtained on the thermospray LC/MS instrument using an isocratic elution of 50% (v/v) methanol in water with 0.1 M ammonium acetate operating at a flow rate of 1 mL/min. The mass spectrometer was operated in the positive ion and filament on mode with the vaporizer tip temperature set to 7 °C below the take-off temperature and a block temperature of 184 °C. A sample of 10 μg/mL each of **1**–**6**, 25 μg/mL **18**, 50 μg/mL each of **17** and **25**–**28**, and 25 μg/mL each of the 2-ketoalkanes (C_4–C_{12}) in 10% methanol in distilled water was prepared, and a 10 μL injection was run to determine their retention times and retention index[26,27] (Table 6).

Antimalarial Activity. The *in vitro* antimalarial activity was determined by Dr. Steve Meshnick, Department of Epidemiology at the University of Michigan School of Public Health, Ann Arbor, MI, using an African (chloroquine sensi-

tive) clone of *P. falciparum* in 1.5% hematocrit cultures. *P. falciparum*, strain FCR-3, was cultivated in candle jars by the method of Trager and Jensen[32] in RPMI 1640 (Gibco Biologicals, Grand Island, NY) containing 10% human serum. Parasites were synchronized by sorbitol lysis[33] for at least three consecutive cycles before use in the experiment. *In vitro* assessment of antimalarial activity of artemisinin, arteether, and its glucuronide derivatives was performed by the microdilution method as previously described.[34] Each of the compounds was serially diluted with medium in a microtiter plate to a final volume of 100 μL. To each well of the microtiter plate was then added 100 μL of parasitized cells (synchronized at ring stage, diluted with normal red cells to 0.5–1% parasitemia, 5–10% hematocrit). The microtiter plate was placed in a candle jar at 37 °C for 24 h. After 24 h incubation, 25 μL of 2 μCi [^{3}H]hypoxanthine in RPMI was added to each well of the microtiter plate and the plate was further incubated for another 24 h. The microtiter plate was harvested using a Brandel cell harvester Model M24 (Brandel, Gaitherburg, MD) and counted on a Beckman LS 7000 scintillation counter (Beckman Instrument, Fullerton, CA) using Scinti Verse BD (Fisher Scientific, Fair Lawn, NJ) as scintillant. Control parasitized cells without drug were performed at the same time. The count represented (as DPM) values for the uptake of hypoxanthine. From the sigmoidal log, dose-response relationship IC_{50} values were determined (Table 7).

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Supplementary Material Available: Summary of R_f and *m/z* values, complete ^{1}H-NMR assignments, and calculated H–H distances (7 pages). Ordering information is given on any current masthead page.

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