Synthesis and Quantitative Analysis of Diastereomeric Linked Ester Conjugates With Remote Stereocenters Using High-Field NMR and Chiral HPLC

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ABSTRACT A stereochemically safe high-yielding procedure for linking unprotected as well as protected hydroxycarboxylic acids to chiral secondary alcohols via glycolic acid linker is proposed. L-menthol has been linked with both enantiomers of mandelic, malic, and methoxyphenylacetic acid using bromo- or iodoacetyl group as a precursor of the glycolic acid linker. High-field nuclear magnetic resonance (NMR) and chiral high-performance liquid chromatography (HPLC) determination of high diastereomeric ratio (*dr*) (>99%) of the products bearing remote stereocenters was explored. Chiral HPLC allowed quantitation of the diastereomers up to *dr* 99.9/0.1. High-field NMR quantitation of the diastereomeric and parent alcoholic impurities in esters was demonstrated at the molar 0.3% and 0.03% levels, respectively. These analyses were done via comparison of integral intensities from major component ^{13}C satellites in ^{1}H or even in ^{13}C spectra to the ^{1}H or ^{13}C signals of impurities. Despite lower sensitivity, the last option generally has much better selectivity. In this way the dynamic resolution is brought down by two orders. *Chirality 25:793–798, 2013.*

KEY WORDS: glycolic acid linker; *O*-alkylation of carboxylic acid; prodrug homogeneity; mandelic acid; lactic acid; malic acid; L-menthol

INTRODUCTION

The use of prodrugs for optimal introduction of a drug molecule into the target site,[1–5] development of their synthesis, and more suitable constituent combinations,[1] especially within frames of mutual prodrug concept,[2] have been of continuous interest.[1–7] The compounds linked into prodrugs have a certain bioactivity by themselves. They are bound together by linker molecules in order to obtain better target properties, such as improved bioavailability[8] and fewer side effects.[7] A variety of moieties to be linked and linkers can be used to achieve the desired construct, ranging from ethers and esters[9,10] to amides.[11] Several prodrugs and biopolymers have been synthesized by linking together chiral hydroxycarboxylic acids via glycolic acid linker.[12–14] The use of chloroacetate as a precursor of glycolic acid linker has been reported.[15] Although linking of bile acids reported is undoubtedly a complex task, the yield of such conjugates (27%) could still be further improved by modifying the process.

Developing methods in three fields: 1) for linking together chiral compounds, 2) for the analysis of diastereomeric ratio (*dr*) of the conjugates gained, and 3) for the determination of enantiomeric ratio (*er*) of chiral starting compounds forms a symbiotic process. For instance, a number of methods based on nuclear magnetic resonance (NMR)[15–25] spectroscopy of diastereomers are used for the determination of *er* and the absolute configuration of chiral compounds.

Diastereomeric homogeneity of the target conjugates depends on both the *er* of the starting compounds as well as on the stability of the constituents that could racemize[26] throughout the linking procedure. The requirements to homogeneity of the target prodrug are stringent; the desired level is usually set at molar 0.1% content of the undesired stereoisomer.[27] This value has to be at least as high as the limit of quantitation (LOQ) for an assured analysis. Works where high-performance liquid chromatography (HPLC)[27–31] or capillary electrophoresis[32,33] (CE) have been used describe particular efforts made in order to improve stereochemical analysis. Chiral HPLC has been routinely used for the determination of *er* of enantiomeric compounds, but less often used for the analysis of diastereomers with remote stereocenters. Nonchiral HPLC of such conjugates is complicated due to the low resolution of the isomers.

The possibility of diastereomer differentiation by NMR spectroscopy generally depends on the number of bonds between the chiral centers.[34,35] The selectivity for ^{13}C nuclei is usually much higher than for ^{1}H. The sensitivity of both ^{1}H and ^{13}C NMR in the analysis of different impurities has often been underestimated. However, recently an interesting work has been published reporting a highly efficient and environmentally benign procedure for fast NMR analysis of small samples of chiral alcohols and amines.[36] The authors derivatize samples directly in the NMR tube. This technique gives to the proposed analytical approach an additional, highly advantageous economical dimension. Furthermore,

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regarding sensitivity of the NMR method, the authors successfully demonstrated measurements of as small as $<10^{-5}$ g of a chiral sample. This significant achievement confirms that, actually, for the quantitative analysis of trace impurities in many systems high-field ^{1}H NMR could be considered more suitable than HPLC or CE, affording comparable or higher sensitivity along with more reliable identification. The use of high-field NMR also results in better selectivity for analysis. In the current work, in order to enhance the reliability of the quantitation, certain products obtained were analyzed using both chiral HPLC and 800/200 MHz ^{1}H/^{13}C NMR. However, the success of analysis of minor impurities using either high field NMR or HPLC always depends on the structure of the diastereomers as well as on the presence of different other trace impurities that may cause overlapping of signals.

MATERIALS AND METHODS

General Experimental Procedures

^{1}H and ^{13}C NMR spectra were recorded in $CDCl_3$ solutions on Bruker Avance 800 and 400 MHz spectrometers. All signals were referenced relative to solvent signal (7.27 ppm for ^{1}H and 77.00 ppm for ^{13}C). 2D FT methods were used for the full assignment of NMR spectra.

Quantitation of trace impurities by ^{1}H spectra. Analytical test procedure: regular 99.9% quality deuterated $CDCl_3$ and a regular quality 5 mm sample tube was used; 128 scans were collected into 32 K data points (acquisition time 2.5 sec) with 40° pulse and 20 sec relaxation delay. Free induction decay (FID) was transformed into128K data points without any window functions.

Quantitation of trace impurities by ^{13}C spectra. Analytical test procedure: on using ^{13}C satellites for the quantitative determination of impurities, signal from about 10 mg of mixture in $CDCl_3$ solution was collected overnight (10,000 scans) into 128 K data points (acquisition time 2.0 sec) with 30° pulse and 2 sec relaxation delay. In order to obtain better resolution at baseline, Gaussian multiplication of FID with LB = -0.2 Hz and GB = 0.7 were used before FID transformation into 256 K data points. For HPLC analysis a Shimadzu Prominence HPLC set was used.

HPLC determination of the enantiomeric ratio of (***S***)-**4**/(***R***)-**4** was performed using a Phenomenex Lux column, eluent: 10% isopropanol/n-hexane (isopropanol contains 0.5% TFA); flow rate, 1.0 ml/min; detection, UV 254 nm; the retention time of the enantiomers: (***S***)-**4** 8.60 min, (***R***)-**4** 10.48 min.

HPLC determination of the diastereomeric ratio of (**14*S***)-**9**/(**14*R***)-**9** was performed using an IA column with chiral stationary phase "amylose tris(3,5-dimethylphenyl-carbamate immobilized on 5 μm silica gel" (Daicel Chiralpak IA; 0.46 cm Ø / 25 cm); eluent, 5% isopropanol/n-hexane; flow rate, 1 ml/min; detection, UV 254 nm; the retention time of the diastereomers: (**14*S***)-**9** 11.06 min, (**14*R***)-**9** 13.51 min.

General Procedure A for linking

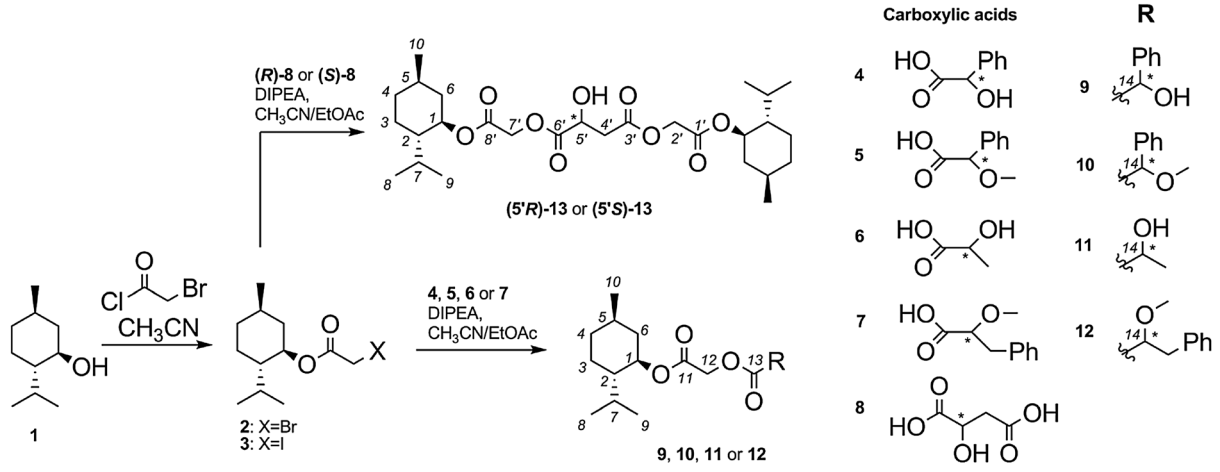
A carboxylic acid was dissolved in acetonitrile (1 vol). *N*,*N*-Diisopropylethylamine (DIPEA) (1.5 eq per one carboxyl group) was added dropwise on stirring. After that, L-(–)-menthyl haloacetate (**2** or **3**) dissolved in EtOAc (1 vol) was added dropwise to the solution of trialkylammonium carboxylate. The reaction progress was monitored by thin-layer chromatography (TLC). After the reaction was complete, the reaction mixture was diluted with EtOAc (50 mL) and the product was washed with 5% solution of $NaHCO_3$ (2 x 15 mL) (the product was also washed with a 5% solution of sodium sulfite (1 x 15 mL) if iodoacetate was linked) and with brine (2 x 15 mL). The solution of the reaction product was then dried on anhydrous Na_2SO_4, filtered, evaporated, and purified by column flash chromatography over silica gel.

General Procedure B for linking

A carboxylic acid was dissolved in acetonitrile (1 vol). *N*,*N*-Diisopropylethylamine (DIPEA) (1.5 eq per one carboxyl group) was added dropwise on stirring. After that, L-(–)-menthyl bromoacetate **2** dissolved in acetonitrile (1 vol) was added dropwise to the solution of trialkylammonium carboxylate. The reaction progress was monitored by TLC. After the reaction was complete the reaction mixture was evaporated on a rotary evaporator and the residue was taken up in EtOAc (50 mL). The product was washed with 5% solution of $NaHCO_3$ (2 x 15 mL) and with brine (2 x 15 mL). The solution of the crude reaction product was then dried on anhydrous Na_2SO_4, filtered, evaporated, and purified by column flash chromatography over silica gel.

RESULTS AND DISCUSSION

Herein we have focused on the base-catalyzed linking of unprotected and protected chiral hydroxycarboxylic acids[37,38] to menthol as a model compound via glycolic acid linker (Scheme 1). A haloacetyl[39,40] group attached to menthol was used as the precursor of the linker. The synthetic goal of the work is the development of a stereochemically safe and technically simple high-yielding protocol for the linking. The analytical goals of the work are the exploration of methods that would allow to determine *dr* of the target conjugates at the content of 0.1–0.3% of the minor diastereomer and the development of a ^{1}H NMR method for quantitation of traces of parent alcohol or other impurities at molar ratio equal to or less than 0.1% in the ester.



Scheme 1. The synthesis of the chiral carboxylic acid conjugates.

TABLE 1. Description of the starting compounds and the syntheses performed; the results of analyses of the products gained

		Carboxylic acid						*Product*			
										dr *determined by*	
Run	*Halo-acetate(mmol)*	*No.*	*mmol*	er % *given*	er*determinedby HPLC*	*Reaction medium*[a]	*Time,h*	*No.*	*Yield%*	*NMR*	*HPLC*
1	*0.4*	(*S*)-*5*	*0.3*	*>99.5*	-	*CH_3CN/EtOAc*	*24*	(*14S*)-*10*	*92*	*n.d.*[c,e]	-
2	*0.4*	(*R*)-*5*	*0.3*	*>99.5*	-	*CH_3CN*	*20*	(*14R*)-*10*	*55*	*n.d.*[c,e]	-
3[b]	*0.4*	(*S*)-*4*	*0.3*	*>99.5*	*n.d.*[c]	*CH_3CN/EtOAc*	*20*	(*14S*)-*9*	*83*	*n.d.*[c,e]	-
4	*0.3*	(*R*)-*4*	*0.2*	*>99.5*	*n.d.*[c]	*CH_3CN*	*24*	(*14R*)-*9*	*38*	*n.d.*[c,e]	-
5	*0.4*	(*S*)-*6*	*0.3*	*>99.5*	-	*CH_3CN/EtOAc*	*20*	(*14S*)-*11*	*88*	*n.d.*[c,e]	-
6	*0.4*	(*rac*)-*6*	*0.6*	*1/1*	-	*CH_3CN*	*6*	(*rac*)-*11*	*39*	*1/1*[e]	-
7	*0.4*	(*R*)-*7*	*0.3*	*~2/1*	-	*CH_3CN/EtOAc*	*48*	(*14R*)-*12*	*91*	*65/35*[e]	-
8[d]	*1.9*	(*S*)-*8*	*0.9*	*99+*	-	*CH_3CN /EtOAc*	*120*	(*5`S*)-*13*	*38*	*n.d.*[c,e]	-
9[d]	*2.1*	(*R*)-*8*	*1.0*	*99+*	-	*CH_3CN /EtOAc*	*120*	(*5`R*)-*13*	*41*	*n.d.*[c,e]	-
10	*0.4*	(*S*)-*4*	*0.3*	*>99.5*	*n.d.*[c]	*CH_3CN /EtOAc*	*24*	(*14S*)-*9*	*86*	*LOD*[f,g]	*99.90/0.10*
11	*0.4*	(*S*)-*4*	*0.3*	*99.0*	*98.95/1.05*	*CH_3CN /EtOAc*	*20*	(*14S*)-*9*	*88*	*98.8/1.2*[f]	*98.91/1.09*
12	*0.4*	(*S*)-*4*	*0.3*	*99.8*	*99.79/0.21*	*CH_3CN /EtOAc*	*20*	(*14S*)-*9*	*74*	*99.7/0.3*[f]	*99.73/0.27*
13	*0.4*	(*S*)-*4*	*0.3*	*99.8*	*99.80/0.20*	*CH_3CN /EtOAc*	*20*	(*14S*)-*9*	*76*	*99.7/0.3*[f]	*99.71/0.29*

General conditions (*modifications are specified*): *reactions were performed at 20 °C using bromoacetate* ***2***.
[a]*Ratio of components in solvents' mixtures was 1/1* (*v/v*).
[b]*Iodoacetate* ***3*** *was used.*
[c]*n.d., other stereoisomer was not detected.*
[d]*Reactions were performed at 55 °C.*
[e]*An ordinary NMR analysis.*
[f]*An estimation using NMR analysis based on the use of integral intensities of* ^{13}C *satellites.*
[g]*The content of the minor diastereomer was comparable to the limit of detection* (*LOD*).

In order to estimate the scope and limitations of the synthetic method proposed, both enantiomers of mandelic acid (MA, 4), malic acid (8), and methoxyphenylacetic acid (MPA, 5) were linked separately to the menthyl bromoacetate (2) (Table 1). In addition, MA was linked in the form of artificial mixtures of enantiomers (Table 1; runs 11–13). Also, lactic acid (6) and 2-methoxy-2-benzylacetic acid (MBA, 7) were linked successfully to bromoacetate 2. Depending on the structure of the carboxylic acid, one significant difference was observed: for the *O*-alkylation of dicarboxylic malic acid (8) a higher temperature and longer reaction time were needed to afford products in satisfactory yield. Neither racemization nor stereochemical discrimination was noted for the compounds in the linking process. Reaction medium CH_3CN/EtOAc (1/1; v/v) is preferable, since it affords higher yields of the products in comparison with neat acetonitrile (Table 1). This could be related to better solubility of menthyl haloacetates 2 and 3 or other components in the mixture of the solvents. The isolated yield of the products gained in the solvent mixture ranges from 74% to 92% (Table 1), while for malic acid (8) the conditions should be optimized.

The suitability of menthyl iodoacetate (3) for linking (Table 1, run 3) was demonstrated. This is important because it extends the scope of the method to chloroacetates as precursors to glycolic acid linker that can be synthesized also by selective enzyme-catalyzed acylation of complex molecules (followed by halogen exchange).

The artificial mixtures of MA enantiomers prepared were analyzed prior to linking, using chiral HPLC. The analysis afforded results (up to 99.8/0.2 *er*) in accordance with the amounts of (*R*)-MA added. The mixtures of MA were linked with bromoacetate 2 and *dr* of the products was analyzed by using chiral HPLC and 800/200 MHz NMR (Table 1, runs 11–13). The change along the *dr*-s determined by chiral HPLC was found to be in line with the differences in the *er* of MA starting samples. The systematic nearby constant difference between the *er* values determined by chiral HPLC for the starting MA samples and the corresponding *dr* values of the products, measured by both chiral HPLC and NMR could be explained by different elution properties of carboxylic acids vs. corresponding esters. This evidently causes an underestimation of the content of the minor R-enantiomer of MA in the samples. The contamination of (*R*)-MA in the commercial (*S*)-MA analyzed in the form of diastereomers 9 was found to be a bit less than 0.1% (see Table 1, run 10). Thus, the LOQ could be estimated to be between 0.1–0.3% of the minor diastereomer (14*R*)-9 in the mixture of diastereomers determined by chiral HPLC.

For the NMR determination of the *dr* the use of ^1H and ^{13}C NMR spectra was explored. Comparison of differential ^{13}C and ^1H shielding effects in some diastereomers in which chiral centers are separated by six (2 C-C and 4 C-O) bonds are presented in Table 2. Much higher selectivity of ^{13}C NMR for the differentiation of diastereomers was observed and can be used for the determination of *dr* in cases with not very different concentrations of diastereomers (e.g., run 7 in Table 1). For the quantitative determination of minute diastereomeric impurities we propose to use the comparison of ^{13}C satellite integrals of ^1H or even ^{13}C NMR spectra and ^1H or ^{13}C signals from the impurities, respectively. In this way the dynamic resolution is brought down by about two orders! The ^{13}C satellite intensity on one side of the main signal is equal to 0.535% in ^1H spectrum and (1–4) × 0.535% in ^{13}C spectrum, the latter depending on the number of carbon atoms directly bonded to the carbon atom under analysis.

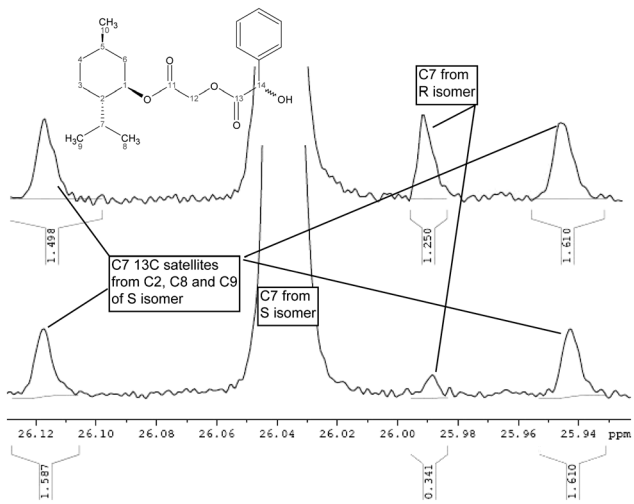


Fig. 1. The use of ^{13}C satellites in 200 MHz ^{13}C NMR spectra for the determination of low concentrations of diastereomers in which chiral centers are separated by six bonds.

TABLE 2. The differential shielding effects in the NMR spectra

		δ differences (*R-S*) between compounds *			
C atom no.		(14*R*)-9 (14*S*)-9	(14*R*)-10 (14*S*)-10	(14*R*)-11 (14*S*)-11	(14*R*)-12 (14*S*)-12
2	^{13}C	+0.018	+0.037	-0.009	+0.009
3	^{13}C	-0.013	+0.017	+0.026	-0.004
6	^{13}C ^1H	-0.021 n.d. n.d.	-0.017 -0.02(eq) n.d.	-0.031 n.d. n.d.	-0.011 0.004(eq) n.d.
7	^{13}C ^1H	-0.050 +0.04	-0.007 +0.04	+0.042 n.d.	+0.027 n.d.
8	^{13}C ^1H	n.d. +0.005	+0.024 +0.009	-0.021 -0.002	n.d. +0.003
9	^{13}C ^1H	+0.021 +0.018	+0.028 +0.027	+0.018 n.d.	-0.012 -0.006
10	^{13}C ^1H	n.d. -0.007	-0.006 -0.010	n.d. n.d.	n.d. +0.001
11	^{13}C	-0.031	-0.004	n.d.	n.d.
12	^{13}C ^1H	-0.020 +0.017 -0.002	0.015 +0.009 -0.002	n.d. +0.002 -0.003	-0.024 +0.003 -0.001
13	^{13}C	-0.059	-0.117	+0.012	-0.012

*n.d., difference was not detected, for ^1H only rows, where differences were observed, are indicated.

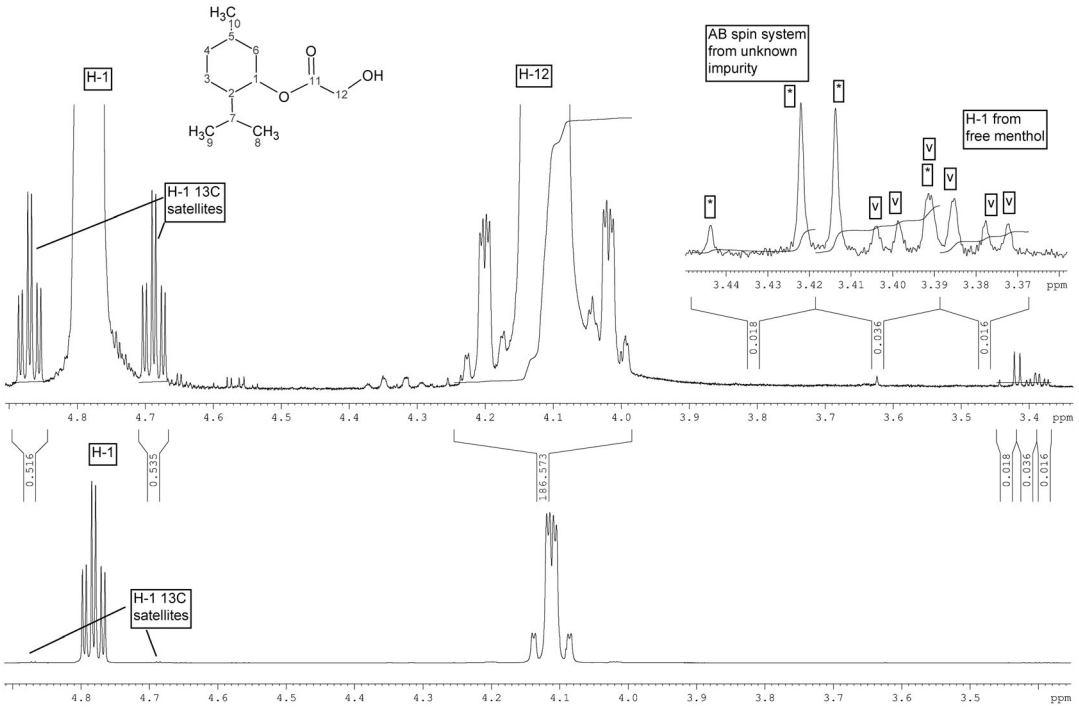


Fig. 2. A precise quantitation of parent menthol at molar 0.03% level in glycolic acid ester by using 800 MHz ^{1}H NMR.

The use of ^{13}C satellites in ^{13}C spectra for quantitative analysis seems to be an unreasonable idea, but it is an option in cases where ^{13}C satellites in ^{1}H NMR spectrum cannot be used. In modern high-field instruments about 10 mg is enough to perform such an analysis for medium-sized molecules. In Figure 1 quantitative analysis of diastereomeric impurity via ^{13}C satellites in ^{13}C NMR spectra of (14*R*)-9/(14*S*)-9 is illustrated. In ^{1}H NMR spectra of diastereomers with remote stereogenic centers it is often hard to find a good integration region for the minor isomer. (For (14*R*)-9/(14*S*)-9 mixture the signal from H14 could be used; but in the current case this position was overlapped by a signal from a minute other impurity.) However, the low concentration of more differing compounds, for instance, parent menthol in menthyl glycolate, can be precisely quantitated at molar 0.03% level by ^{1}H high-field NMR even despite partially overlapping signals from H1 of free menthol and from an additional trace impurity (Fig. 2).

CONCLUSIONS

1. A stereochemically safe high-yielding protocol for linking unprotected and protected hydroxycarboxylic acids to chiral secondary alcohols via glycolic acid linker has been proposed.
2. Independent analytical methods: using high-field NMR as well as chiral HPLC for quantitation of trace diastereomeric impurities in the conjugates with remote stereocenters were proposed.
3. An NMR approach based on the use of ^{13}C satellites in ^{13}C spectra gives precise estimations for *dr* at about 1.0% content of the minor diastereomer and less precise but quite adequate and reliable (regarding identification) estimations for diastereomeric contaminations at 0.3–0.7% (Table 1, runs 12 and 13).
4. A sensitive high-field ^{1}H NMR technique for quantitation of parent alcoholic impurity in ester at LOQ = molar 0.03% has been demonstrated.

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