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Chromatographic Determination of the Absolute Configuration of an Acyclic Secondary Alcohol Using Difluorodinitrobenzene

Ken-ichi Harada,* Youhei Shimizu, Atsuko Kawakami, Makiko Norimoto, and Kiyonaga Fujii

Faculty of Pharmacy, Meijo University, Tempaku, Nagoya 468-8503, Japan

A chromatographic determination method was developed for the absolute configuration of an acyclic secondary alcohol using the characteristic functions of 1,5-difluoro-2,4-dinitrobenzene (FFDNB). This method relies on the formation of the fixed favorable conformation of a secondary alcohol-DLA (2,4-dinitrophenyl-5-leucinamide) derivative and its recognition by ODS silica gel. The secondary alcohol reacted first with FFDNB under mild basic conditions, and L-leucinamide or DL-leucinamide was then introduced into the secondary alcohol-FDNB derivative. Because the conformations of the resulting alcohol-DLA derivatives were rigidly fixed by the dinitrobenzene plane, the absolute configuration at the asymmetric carbon of the secondary alcohol tested can be definitely deduced by the elution behavior of both of the diastereomers in the HPLC and/or LC/MS. One of the diastereomers has the cis (*Z*)-type arrangement of two more hydrophobic substituents of the alcohol and leucinamide moieties, the more hydrophobic side chain and isobutyl groups, to the plane of the dinitrobenzene, whereas another diastereomer has the opposite arrangement (trans (*E*)-type). Therefore, the cis arrangement interacts more strongly with the ODS silica gel and has a longer retention time than that of the trans-type arrangement. This established method was successfully applied to various chiral acyclic secondary alcohols including chloramphenicol and 4-hydroxyphenyllactic acid. Finally, the limitations of this method were also examined.

Although there are several determination methods such as X-ray analysis, circular dichroism (CD), and NMR spectral techniques for the determination of the absolute configuration of secondary alcohols, a modified Mosher's method has been widely used for the past decade because of its universality and accessibility.[1] This method is based on the derivatization of a compound to be tested with the two enantiomers of a chiral anisotropic reagent, methoxytrifluoromethylphenylacetic acid (MTPA) and comparison of the chemical shifts of the resulting diastereomers. This methodology using NMR spectroscopy can be regarded to consist of two processes, the freezing of the conformation of a resulting diastereomer and the recognition of the desired conformation based on the influence of the NMR anisotropy effect. However, some of the drawbacks of this method were pointed out. To the best of our knowledge, no suitable method has been developed for the first process; therefore, one must always discuss the conformation of the resulting diastereomer. For example, Riguera et al. pointed out that MTPA esters are constituted by three main conformers in close populations and that methoxyphenylacetic acid (MPA) is superior to MTPA based on the results of extensive conformational studies.[2] Although they recommended a single derivatization method using MPA in combination with low-temperature measurement, they also needed to discuss the detailed conformation.[3]

The "advanced Marfey's method" has been developed to nonempirically determine the absolute configuration of constituent amino acids in a peptide using LC/MS.[4,5] This method consists of Marfey's method[6] as a chromatographic technique for the separation of amino acids into each enantiomer, the detection of the amino acid by mass spectrometry, and a procedure for obtaining the corresponding enantiomer from either the L- or D-amino acid. In this method, the resolution between the L- and D-amino acids derivatized with 1-fluoro-2,4-dinitrophenyl-5-L-leucinamide (L-FDLA) is due to the difference in their hydrophobicity, which is derived from the cis (*Z*)-type or trans (*E*)-type arrangement of two more hydrophobic substituents. Therefore, the L-amino acid is usually eluted before the corresponding D-amino acid.[7] The separation mechanism for the resolution of both resulting diastereomers is based on a rigidly fixed conformation assisted by the intramolecular hydrogen bonding between the nitro groups and α-amino groups of the amino acid and leucinamide moiety. In addition to this method, DL-FDLA derivatization was introduced for obtaining the corresponding enantiomer on

* Corresponding author: (tel) 81-52-832-1781; (fax) 81−52−834−8780; (e-mail:) kiharada@meijo-u.ac.jp.

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the chromatogram,[8] and its utility has been successfully extended from amino acids to primary amines.[9]

In a previous paper, we demonstrated that an NMR spectroscopic method using the anisotropy effect can determine the absolute configuration of the α-carbon of a secondary alcohol.[10] In this method, a secondary alcohol was derivatized first with 2,4-dinitro-1,5-difluorobenzene (FFDNB) to yield an alcohol-FDNB derivative, which led to the desired 2,4-dinitrophenyl-5-phenylethylamine (DPEA) derivative after derivatization with 1-phenylethylamine. The resulting conformation of the alcohol-DPEA derivative was fixed by means of the dinitrobenzene plane and was quite similar to those of the 2,4-dinitrophenyl-5-leucinamide (DLA) derivatives of the primary amines including the amino acids mentioned above. Consequently, the configuration of a secondary alcohol was effectively determined by this method. These results strongly indicate that the same methodology can be applied to the case when HPLC is used as the recognition method instead of NMR spectroscopy. In this paper, we describe a procedure for the determination of the configuration of an acyclic secondary alcohol using HPLC and/or LC/MS.

EXPERIMENTAL SECTION

Chemicals. D- and L-hydroxyphenyllactic acid (Hpla) were generous gifts from Dr. M. Murakami (Graduate School of Agricultural and Life Sciences, The University of Tokyo, Japan). FFDNB, leucinic acid, 1-aminopentane, and 2-aminophenylethane were purchased from Tokyo Kasei Co., Tokyo, Japan. Triethylamine and chloramphenicol were obtained from Nacalai Tesque, Kyoto, Japan. Leu and DL-Hpla were purchased from Sigma, St. Louis, MO. Leucine amide was obtained from Kokusan Chemicals, Tokyo, Japan. 2-Hydroxybutane and 2-hydroxyhexane were purchased from Aldrich, Milwaukee, WI. 2-Aminobutane, 2-aminopentane, 2-aminoheptane, and 1-aminophenylpropane were obtained from Hydrus Chemical, Inc., Japan. 2-Hydroxyheptane, 2-hydroxyoctane, and 1-hydroxyphenylethane were purchased from AZmax, Kisarazu, Japan. All other reagents and solvents were of the analytical grade.

Preparation of the DLA Derivative of a Secondary Alcohol. The secondary alcohol tested was dissolved in dichloromethane and triethylamine at 40 °C and FFDNB was added to the solution, which was then allowed to stand for 24 h. The reaction mixture was evaporated to dryness, and the residue was applied to an ODS cartridge to remove excess reagent. The cartridge was washed with 35% acetonitrile (aq) and the desired 2,4-dinitro-5-fluorobenzene (FDNB) derivative was eluted with acetonitrile. The eluate was subjected to preparative TLC using an appropriate solvent system. The FDNB derivative reacted with L-leucinamide in 1% triethylamine−acetonitrile at 40 °C for 1 h, and the reaction mixture was evaporated to dryness. The residue was cleaned with a silica gel cartridge, and the desired DLA derivative was then subjected to HPLC or LC/MS.

Spectral Measurement. ^{1}H NMR and NOE difference spectra were recorded on a JNM-A 400 (JEOL, Tokyo, Japan) spectrometer. ^{1}H chemical shifts were referenced to the residual methanol-d_4 signal (δ 3.30 ppm) and DMSO-d_6 (δ 2.49 ppm). UV spectra were obtained using a Shimadzu (Kyoto, Japan) UV-2100 or a model 991J photodiode array detector during HPLC operation described below.

Analysis by HPLC and LC/MS. HPLC was performed using a Tosoh (Tokyo, Japan) dual-pump delivery system. Separation was carried out on a TSK gel ODS-80Ts column (150 × 4.6 mm i.d., Tosoh) maintained at 40 °C. Acetonitrile−0.01 M trifluoroacetic acid (TFA) was used as the mobile phase under a linear gradient elution mode (acetonitrile, 30−80%, 50 min). The flow rate was 1 mL/min with UV detection at 340 nm and 250−500 nm by photodiode array detection. LC/MS was performed below. The separation was carried out on a Develosil ODS-HG-5 column (150 × 2.0 mm i.d., Nomura Chemical, Seto, Japan) maintained at 40 °C using a HP1050 (Hewlett-Packard, Novi, MI). Acetonitrile−water containing 0.01 M TFA was used as the mobile phase under a linear gradient elution mode at a flow rate of 0.2 mL/min. The mass spectrometer was a Finnigan TSQ7000 (Finnigan-Mat, San Jose, CA). All mass spectra were aquired using Q1 as the scanning quadrupole. The ESI voltage was 4.5 kV with the auxiliary and sheath gas nitrogen pressure set at 5 units and 70 psi, respectively, and the capillary was heated to 200 °C.

RESULTS AND DISCUSSION

Although a secondary alcohol was directly derivatized with FDLA, the desired secondary alcohol derivative could not be prepared. On the basis of the reactivity of FFDNB, the reaction sequence was changed to obtain the desired secondary alcohol derivative. That is, the tested secondary alcohol reacted first with FFDNB to yield quantitatively the secondary alcohol-FDNB derivative, and then leucinamide was introduced into the resulting secondary alcohol-FDNB for the recognition (Figure 1). The derivatization procedure for secondary alcohols was finally optimized as follows: the secondary alcohol reacted with FFDNB and triethylamine (molar ratio of 1:8:8) in dichloromethane at 40 °C for 24 h. To remove the excess FFDNB, the reaction mixture was applied to an ODS silica gel cartridge. The resulting secondary alcohol-FDNB derivative reacted with leucinamide in 1% triethylamine−acetonitrile at 40 °C for 1 h. After elimination of the excess reagents, the desired DLA derivatives were subjected to ordinary HPLC analysis under reversed-phase conditions.

To confirm the conformations of the resulting alcohol-FDNB and -DLA derivatives, the nuclear Overhauser effect (NOE) experiments were performed. In the difference NOE spectrum of the (*R*)-1-hydroxyphenylethane-FDNB derivative as the typical chiral secondary alcohol, a strong NOE (12.8%) was observed between the H-6 of FDNB and the α-proton of the secondary alcohol. This spectral behavior is consistent with that of (*R*)-1-aminophenylethane, in which the strong NOE (11. 5%) was also observed between the H-6 of FDNB and the α-proton of the primary amine. These experiments indicated that the α-proton of the alcohol is spatially located near H-6 of the benzene ring and the conformations of the alcohol and amine are very similar. The same results were obtained from the NOE experiment on the DLA derivatives of (*R*)-1-hydroxyphenylethane and (*R*)-1-aminophenylethane. Namely, strong NOEs (∼15%) were observed between the α-protons of the alcohol or amine tested and leucinamide of DLA, and the H-6 of the benzene ring (Figure 2), indicating that

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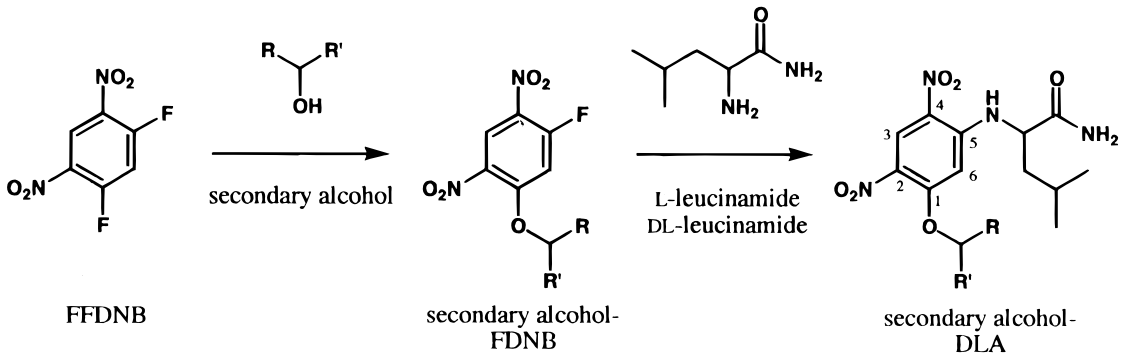


Figure 1. Derivatization procedure for determination of the absolute configuration of an acyclic secondary alcohol.

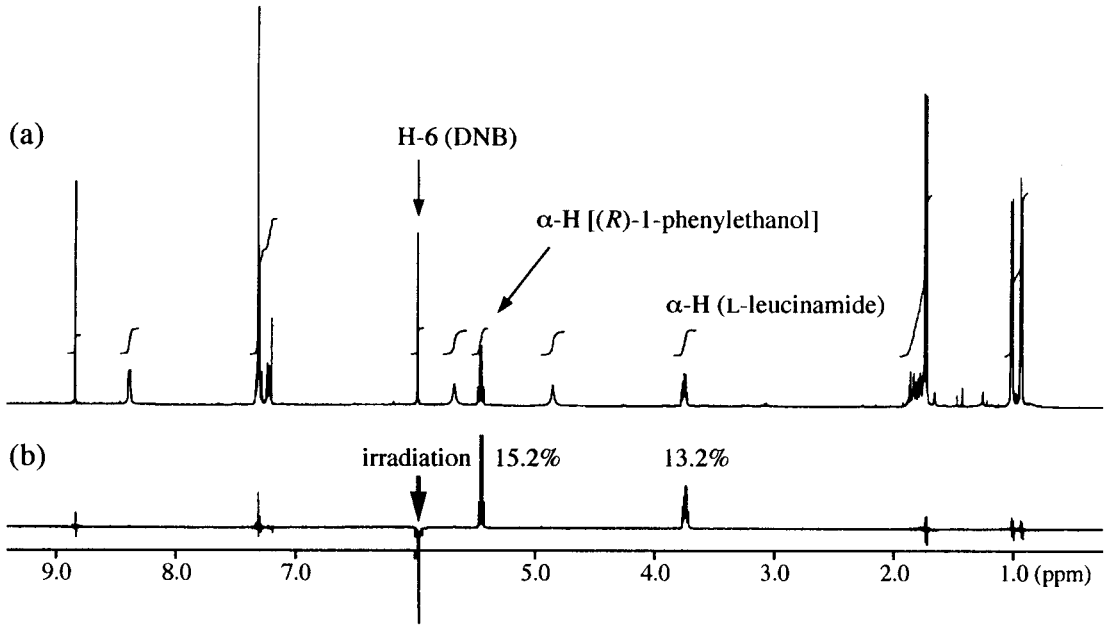


Figure 2. (a) ^{1}H NMR and (b) difference NOE spectra of the DLA derivative of (*R*)-1-hydroxyphenylethane.

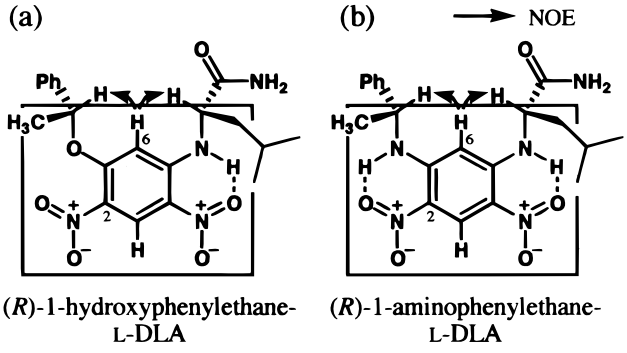


Figure 3. Predominant conformations of the DLA derivatives of (a) (*R*)-1-hydroxyphenylethane and (b) (*R*)-1-aminophenylethane obtained from the NOE experiment.

both of the α-protons are spatially situated near H-6 of the benzene ring in the alcohol-DLA derivative (Figure 3). In addition, we had confirmed that this predominant conformation is also formed in the case of more complicated cyclic alcohols.[10]

The UV spectral method was very useful for examining the desired conformation for the resolution of the primary amine derivatized with FDLA.[7] The UV spectrum of (*R*)-1-aminophenylethane-DLA shows the characteristic spectral behavior including the absorption maximums at 415 and 340 nm (Figure 4a). When the primary amine-DLA has the desired conformation for the resolution, its spectrum always shows clearly the two absorption maximums, which are derived from the bridge chromophore between the nitro groups of the nitrobenzene and the amino groups of the primary amine and L-leucinamide.[7] However, the UV spectrum of (*R*)-1-hydroxyphenylethane-DLA is characterized by two shoulders at 390 and 320 nm and an absorption maximum at 290 nm (Figure 4c) and is completely different from that of the primary amine-DLA. This spectral behavior was similar to that of the superposition of FDLA (λ_{max} 340 nm, $\lambda_{shoulder}$ 390 nm) (Figure 4b) and (*R*)-1-hydroxyphenylethane-FDNB (λ_{max} 280 nm) (Figure 4d), indicating that the absorption maximum at 415 nm in the UV spectrum of the primary amine-DLA is due to the hydrogen bonding between the nitro group at C-2 of the nitrobenzene and the amino group of the introduced primary amine. The appearance of this absorption maximum depends on the presence or absence of the second hydrogen bonding. Although this hydrogen bonding is absent in the case of the secondary alcohol-DLA, its conformation is rigidly fixed as shown in Figure 3, which was confirmed by the NOE experiment. Because the UV spectrum is readily available using a photodiode array detector during HPLC analysis, it is useful to check the desired conformation.

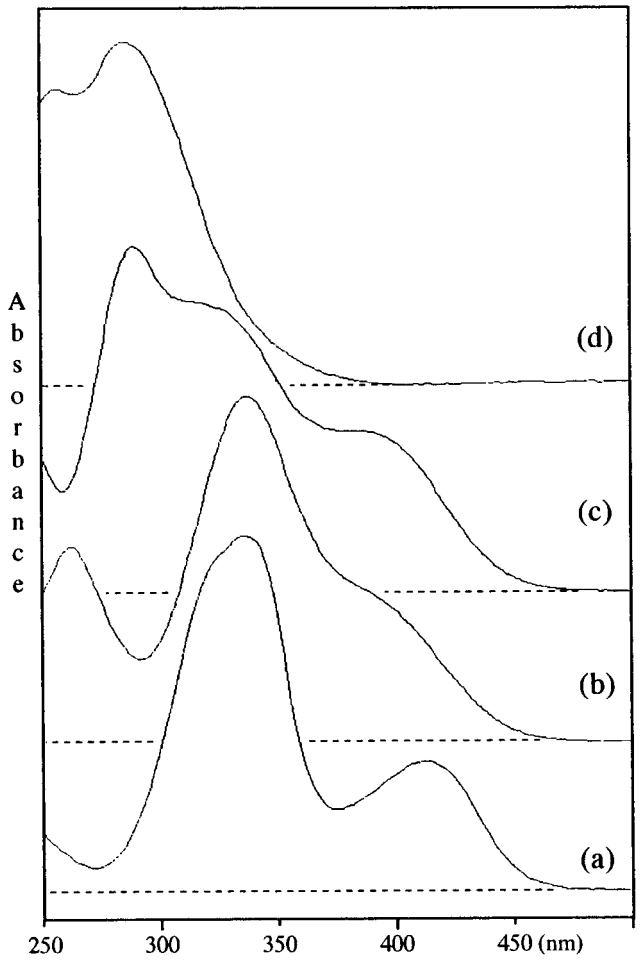


Figure 4. UV spectra of the DLA derivatives of (a) (*R*)-1-aminophenylethane and (c) (*R*)-1-hydroxyphenylethane and (b) FDLA and (d) the FDNB derivative of (*R*)-1-hydroxyphenylethane.

As mentioned above, this conformation of the secondary alcohol derivative was consistent with the case of the primary amine including amino acids (Figure 3). Therefore, the same separation mechanism as that of the primary amine can be also depicted for the secondary-DLA as shown in Figure 5. For example, in the case of 2-hydroxyalkanes, the (*S*)-diastereomer has a cis (*Z*)-type arrangement of the two more hydrophobic substituents of the alcohol and L-leucinamide, the R_1, and isobutyl groups, relative to the plane of the dinitrobenzene, whereas the (*R*)-diastereomer has the opposite arrangement (trans (*E*)-type) (Figure 5). Therefore, the cis arrangement interacts more strongly with the ODS silica gel and has a longer retention time than that of the trans-type arrangement.[7]

This method was first applied to leucine and leucinic acid to examine the chromatographic behavior of the DLA derivative of amino acid and its corresponding alcohol. We had shown that the DLA derivative of a hydrophobic neutral amino acid can be well resolved using this method. Indeed, the peaks of the derivatives of L- and D-leucine appeared at 19.6 and 26.9 min, respectively, on the chromatogram. Further, the derivatives of L- and D-leucinic acid were eluted at 19.2 and 26.2 min, respectively, on the chromatogram. This chromatographic behavior including the retention time and the time difference of both isomers of leucinic acid was closely similar to that of leucine. Subsequently, several acyclic secondary alcohols were examined together with the corresponding primary amines. Table 1 shows the elution order, retention time, and time difference in the HPLC analysis data of (*R*)- and (*S*)-2-hydroxyalkane-DLA and the corresponding primary amine-DLA derivatives under the reversed-phase conditions. All (*R*)-diastereomers of the alcohols tested were eluted prior to the (*S*)-diastereomers without exception. The resolution became better with an increase in the length of the alkyl chains, and the retention time also became longer. This chromatographic behavior for the alcohols was almost the same as that of the primary amines as shown in Table 1. These results suggested that both diastereomers of the alcohols can be resolved through the common mechanism for the primary amines.[7, 9]

The "DL-FDLA derivatization" was developed for obtaining the corresponding enantiomer from an original chiral compound and has been applied to primary amines including amino acids.[5,8] Namely, this method can form a peak of the corresponding enantiomer of a chiral compound on the chromatogram using a simple operation, even if the corresponding enatiomer is not available. Experimentally, a chiral compound was divided into two portions and the two portions were separately derivatized with L-FDLA and DL-FDLA. Both of the derivatives were subjected to HPLC or LC/MS analysis, and comparison of the original peaks with the two peaks can determine the correct absolute configuration. This procedure was slightly modified because the reaction sequence was changed as mentioned above. After the introduction

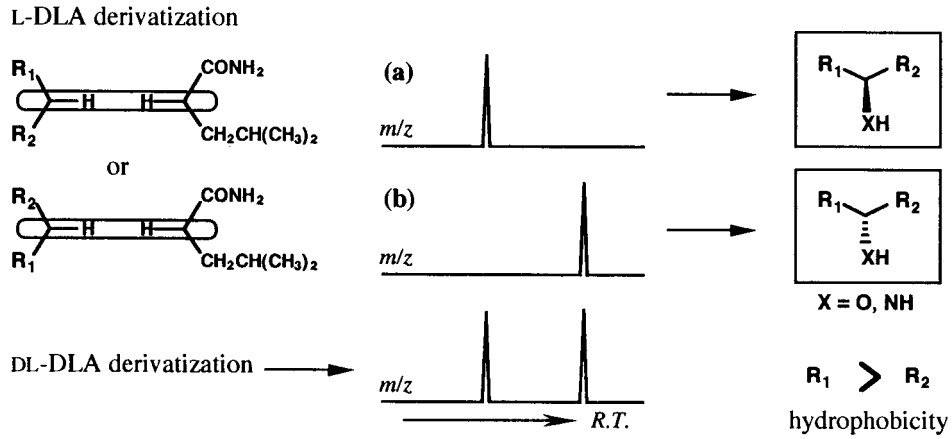
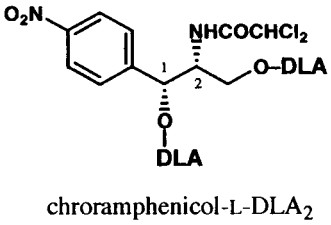


Figure 5. Separation mechanism for the DLA derivatives of secondary alcohols using HPLC.

Table 1. HPLC Analysis Data of Secondary Alcohol-DLA and the Corresponding Primary Amine-DLA Derivatives[a].

	x = OH				x = NH_2			
DLA derivatives	elution order	$t_R(R)$	$t_R(S)$	Δt_R	elution order	$t_R(R)$	$t_R(S)$	Δt_R
2-x-butane	(*R*) → (*S*)	21.5	21.8	0.3	(*R*) → (*S*)	22.6	23.1	0.5
2-x-pentane	(*R*) → (*S*)	25.0	25.7	0.7	(*R*) → (*S*)	26.1	27.1	1.0
2-x-hexane	(*R*) → (*S*)	28.4	29.5	1.1				
2-x-heptane	(*R*) → (*S*)	31.7	33.1	1.4	(*R*) → (*S*)	33.2	34.9	1.7
2-x-octane	(*R*) → (*S*)	35.1	36.6	1.5				
1-x-phenylethane	(*R*) → (*S*)	23.9	25.9	2.0	(*R*) → (*S*)	24.3	26.5	2.2
1-x-phenylpropane	(*R*) → (*S*)	27.2	29.1	1.9	(*R*) → (*S*)	27.6	29.5	1.9

[a] t_R, retention time (min); Δt_R, $t_R(S) - t_R(R)$ (min).



chloramphenicol-L-DLA_2

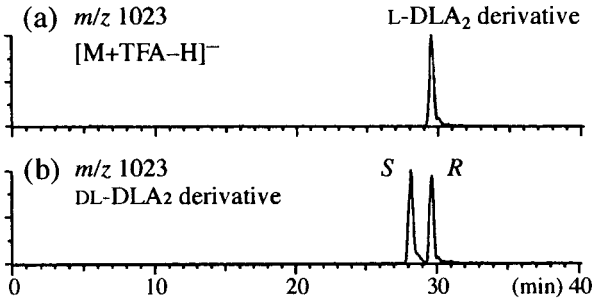


Figure 6. Mass chromatograms at *m*/*z* 1023 [M + TFA − H]⁻ of the (a) L- and (b) DL-DLA derivatives of chloramphenicol analyzed using ESI LC/MS in the negative ion mode.

of an (*S*)-2-hydroxyalkane to FFDNB, the resulting FDNB derivative was derivatized with L-leucinamide and DL-leucinamide and it was confirmed that the peak of the L-DLA derivative of (*S*)-2-hydroxyalkane is completely identical with that of D-derivative of (*R*)-2-hydroxyalkane. This chromatographic behavior was consistent with that of the corresponding amino compound, indicating that this procedure is applicable to a chiral secondary alcohol.[9]

To confirm the utility of this method including "DL-FDLA derivatization", it was applied to chloramphenicol produced by *Streptomyces venezuelae*.[11] This antibiotic reacted with FFDNB, and the resulting FDNB derivative was divided into two portions. Each portion was separately derivatized with L- or DL-leucinamide, and the products were analyzed using LC/MS. The mass chromtograms monitored at *m*/*z* 1023 [M + TFA − H]⁻ are shown in Figure 6, and the D-DLA derivative was eluted prior to the L-DLA one. Because the benzene moiety is more hydrophobic than the side chain, the absolute configuration at C-1 can be determined as *R* based on the conformation shown in Figure 5. This result was consistent with the absolute configuration confirmed previously.

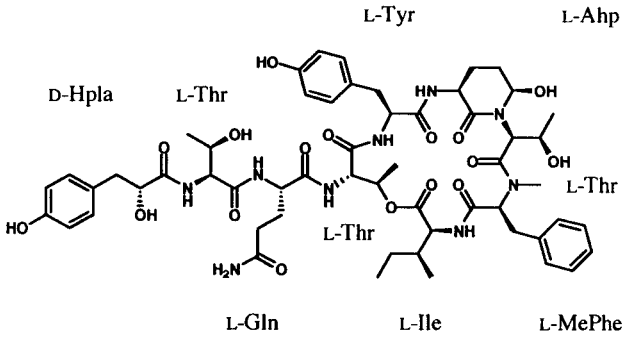


Figure 7. Structure of aeruginopeptin 95A.

Recently, many peptides were isolated from terrestrial cyanobacteria[12] and classified into several groups, one of which is a 19-membered cyclic depsipeptide possessing a 3-amino-6-hydroxy-2-piperidone (Ahp) moiety. The structure of a typical compound, aeruginopeptin 95A, is shown in Figure 7.[13] Some compounds in this group have a Hpla moiety as the *N*-terminal blocking group. The established method was applied to the determination of the absolute configuration of Hpla. Aeruginopeptin 95A was hydrolyzed under the usual conditions, and the reaction mixture was partitioned with diethyl ether and water. The residue from the organic layer was subjected to the derivatization procedure, and the resulting L- and DL-DLA derivatives were analyzed under the LC/MS conditions. Figure 8 shows the mass chromatograms monitored at *m*/*z* 769 [M − H]⁻ of both of the derivatives. Because the D-DLA isomer was eluted prior to the L-DLA isomer, the absolute configuration was determined as D (*R*). This conclusion coincided with that by Shimizu et al.[14] and was also confirmed by comparison of the derivative of authentic samples.

To our knowledge, there is only one method using HPLC, the modified Horeau method,[15] for the determination of the absolute configuration of a chiral secondary alcohol. This method relies on the kinetic resolution of racemic 2-phenylbutyric anhydride by the chiral secondary alcohol. Because the absolute configuration was deduced using the ratio of diastereometric *N*-[1-(1-naphthyl)ethyl]-2-phenylbutanamides determined by HPLC, the results were sometimes ambiguous. Indeed, this method had not been used; instead, a modified Mosher method was developed

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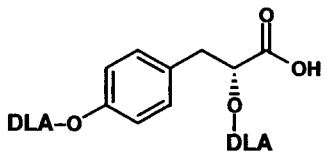

Hpla-L-DLA$_2$

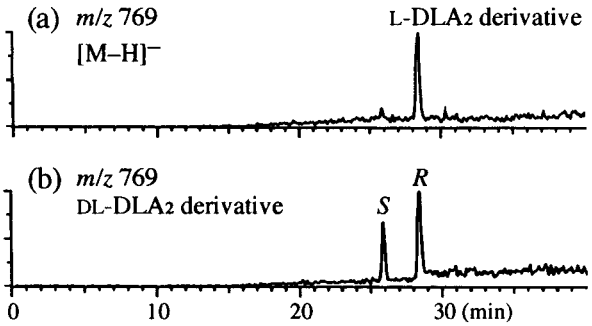

Figure 8. Mass chromatograms at *m*/*z* 769 [M − H]$^-$ of the (a) L- and (b) DL-DLA derivatives of Hpla analyzed using ESI LC/MS in the negative ion mode.

and has been widely used since early 1990.[1] To use this method, we must prepare at least 1 mg of both of the MTPA derivatives of a secondary alcohol, must assign any ^{1}H signals of the derivative, and then must calculate the difference in the chemical shift of each proton in the both isomers. Therefore, this method is not sensitive and its procedure is time-consuming. Further, this method does not often give a definite conclusion because of undesirable conformation. In such a case, a detailed conformation analysis is required.

In the present study, we developed a chromatographic method for the determination of the absolute configuration of an acyclic secondary alcohol as mentioned above. This method is based on the formation of the fixed favorable conformation of a secondary alcohol-DLA derivative and its recognition by ODS silica gel, which can be applied to acyclic secondary alcohols with the aid of mass spectrometry in the same manner as in the case of primary amines. Usually, 1 nmol of the DLA derivative can be detected by this method. Therefore, the method is sensitive and its procedure is easy to handle. However, there are still three problems to be resolved in this method at present. Although a preliminary method was proposed for estimating the hydrophobicity of R_1 and R_2 (Figure 5),[9] no appropriate method has been established so far. Second, it is difficult to apply this method to a cyclic secondary alcohol because it is impossible to estimate its hydrophobicity. For example, the L- and D-DLA derivative of (−)-menthol could be separated (data not shown), but it was hard to predict the correct configuration based on our separation mechanism. Third, we have to shorten the total analysis time of the present method because it takes a whole day to do the first derivatization. The improvement of the derivatization is in progress.

CONCLUSIONS

In the present study, we tried to establish a nonempirical determination method for the absolute configurations of an acyclic secondary alcohol using the characteristic functions of FFDNB in combination with the usual reversed-phase HPLC. This method relies on the formation of the fixed favorable conformation of a secondary alcohol-DLA derivative and its recognition by ODS silica gel. As a result of extensive experiments, it was found that this method can be successfully applied to an acyclic secondary alcohol in the same manner as in the case of primary amines. Through the previous and present studies, we have developed a total system for the determination of the absolute configuration of secondary alcohols and primary amines, in which a fixed conformation is formed using the characteristic functions of FFDNB and the resulting conformation is recognized by NMR spectroscopy or HPLC. The methodology is being further extended.

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