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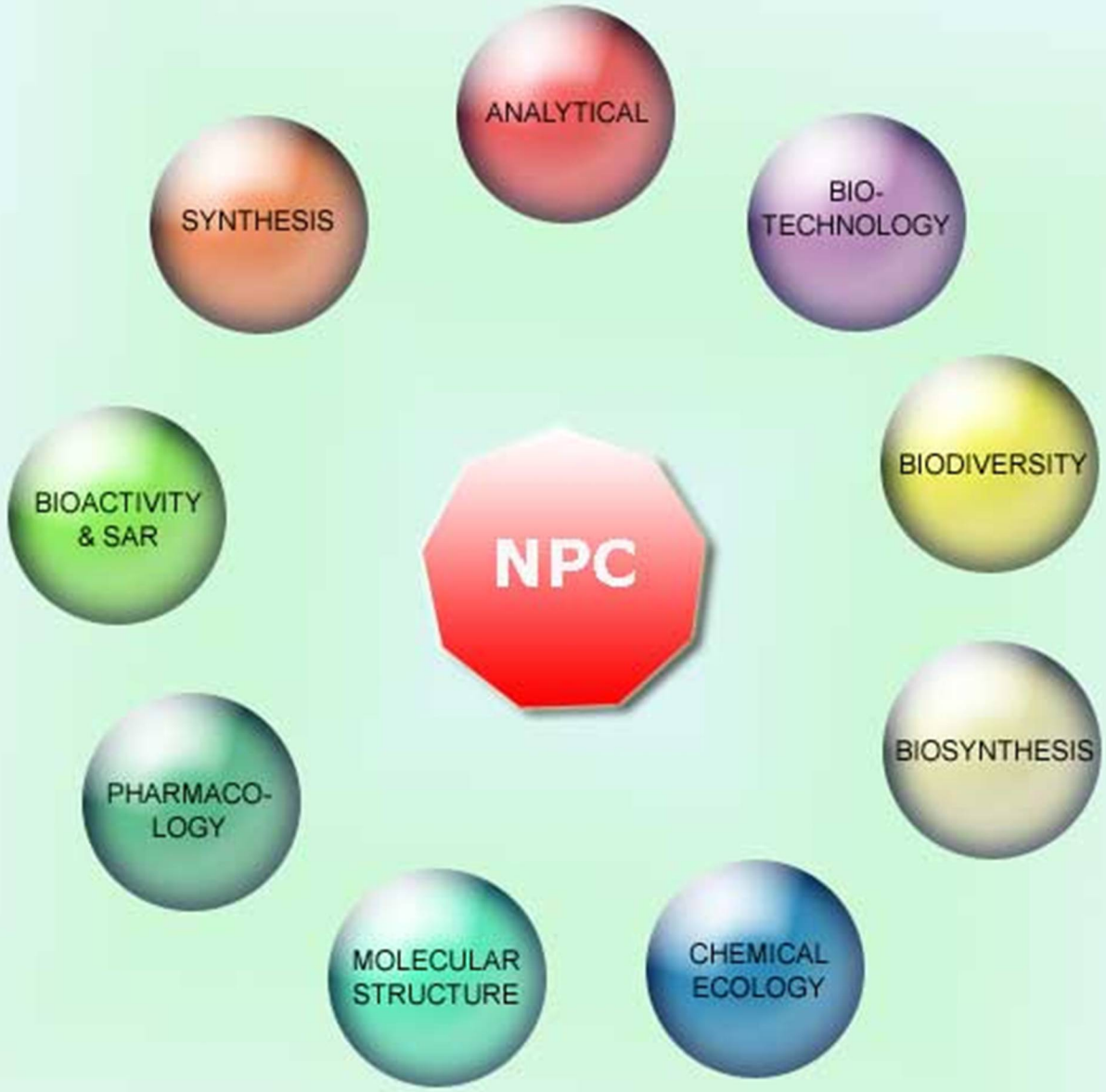


Volume 6. Issue 12. Pages 1799-1968. 2011
ISSN 1934-578X (printed); ISSN 1555-9475 (online)
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NPC Natural Product Communications

2011
Vol. 6
No. 12
1889 - 1892

# Antifungal Activity and Isomerization of Octadecyl *p*-coumarates from *Ipomoea carnea* subsp. *fistulosa*

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**Received: June 6th, 2011; Accepted: October 25th, 2011**

Bioassay monitored HPLC assisted isolation and purification of the chief antifungal fraction of the leaves of *Ipomoea carnea* subsp. *fistulosa* (Convulvulaceae) were achieved using *Colletotrichum gloeosporioides* and *Cladosporium cucumerinum* as test organisms. The activity of the purified fraction was further confirmed by the dose dependent inhibition of the spore germination of *Alternaria alternata* and *A. porri.* The active fraction was identified as a mixture of (*E*)-octadecyl *p*-coumarate and (*Z*)-octadecyl *p*-coumarate. The two isomers were detected on an HPLC column with substantially different retention times, but once eluted from the column, one form was partly converted to the other in daylight. Conclusive evidence for the structures and their isomerization were obtained from the HPLC behavior, IR, UV, HRESIMS, CIMS and NMR spectral data. Important $^{1}$H NMR and $^{13}$C NMR signals could be separately assigned for the isomers using 2D NMR techniques.

**Keywords:** *Ipomoea carnea*, *Ipomoea fistulosa*, Convulvulaceae, antifungal, (*E*) octadecyl *p*-coumarate, (*Z*) octadecyl *p*-coumarate, isomerization, 2D NMR techniques.

*Ipomoea carnea* subsp. *fistulosa* (Mart. ex Choisy) D.F.Austin (Convulvulaceae) is a plant native to South America, but sparsely distributed in India and Bangladesh. It is used in hedgerows along cattle crossings, to fight erosion and as an ornamental. Isolation and chemical characterization of resinous glycosides [1], flavonol glycosides [2] and alkaloids [3] from the leaves, and anthocyanin from the flowers [4] of *I. carnea* have been reported. The leaves are toxic to cattle and the toxicity is attributed to polyhydroxy alkaloids such as swainsonine and calystegines [5]. Recently, a chitinase has been identified in the plant [6]. Antibacterial and antifungal activities of the extractives of the plant have been reported [7], but bioassay monitored isolation and characterizations of the antifungal compounds present in the plant have not yet been carried out. We hereby report the bioassay monitored isolation and characterization of the chief antifungal fraction. The fraction was isolated using *Colletotrichum gloeosporioides* and *Cladosporium cucumerinum* as test organisms and the activity was further confirmed against the spore germination of *Alternaria alternata* and *A. porri.* The active fraction was found to be a mixture of (*E*)-octadecyl *p*-coumarate and (*Z*)-octadecyl *p*-coumarate. The two isomers were detected on the HPLC column with substantially different retention times, but once eluted from the column; one form was partly converted to the other. Conclusive evidence for the structures and their isomerization were obtained from the HPLC behavior, IR, UV, HRESIMS, CIMS, $^{1}$H NMR, $^{13}$C NMR, DEPT and 2D NMR spectral data [8]. Survey of the literature showed several reports on the isolation of octadecyl *p*-coumarates without any mention of its antifungal activity [9]. In all these studies, characterization was achieved without resorting to detailed analysis of $^{13}$C NMR, DEPT and 2D NMR spectra and, for the same reason, isomerization of the (*E*) and (*Z*) forms was not reported. The sole report on antifungal activity [10] is about a mixture of stearyl esters and not of any individual compound.

Thus, this is the first report on the antifungal activity and isomerization of octadecyl *p*-coumarates. This is also the first report in which important $^{1}$H and $^{13}$C NMR signals have been separately assigned for the (*E*) and (*Z*) isomers of octadecyl *p*-coumarates (Table 1). This may also be the first report in which HSQC data have been used to confirm the isomerization of alkyl *p*-coumarates.

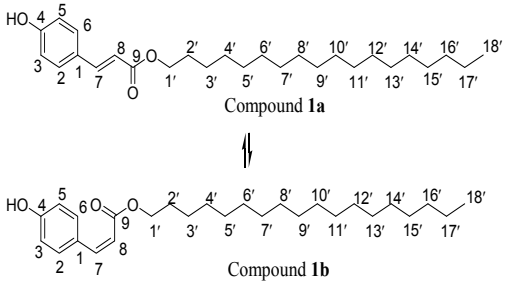


**Figure 1**: Isomerization of (*E*)-octadecyl-*p*-coumarate (**1a)** and (*Z*)-octadecyl-*p*-coumarate (**1b**).

Antifungal activity assay of the crude extractives showed that the ethyl acetate extractive possessed highest activity. Column chromatography of this revealed that the most active fraction was that obtained by elution with *n*-hexane-

**Table 1**: NMR spectroscopic data (400 MHz, $CDCl_3$) of *E*-octadecyl *p*-coumarate (**1a**) and *Z*-octadecyl *p*-coumarate (**1b**).

| Sl. No. | $\delta_C$, mult | Carbon number in the figure | $\delta_H$, mult. (*J* in Hz) | Assigned to *E/Z*/Both |
|---|---|---|---|---|
| 1. | 167.56 C | 9 | - | *E* |
| 2. | 166.73 C | 9 | - | *Z* |
| 3. | 157.59 C | 4 | - | *E* |
| 4. | 156.62 C | 4 | - | *Z* |
| 5. | 144.22 CH | 7 | 7.62,d, *J*=12.8 Hz | *E* |
| 6. | 143.18 CH | 7 | 6.84, d, *J*=10.0 Hz | *Z* |
| 7. | 132.30 CH | 6,2 | 7.63,d, *J*= 6.8 Hz | *Z* |
| 8. | 129.92 CH | 6,2 | 7.43, d, *J*=6.8 Hz | *E* |
| 9. | 127.52 C | 1 | - | *Z* |
| 10. | 127.35 C | 1 | - | *E* |
| 11. | 117.33 CH | 8 | 5.83,d, *J*=10.0 Hz | *Z* |
| 12. | 115.84 CH | 3,5 | 6.84, d, *J*=6.8 Hz | *E* |
| 13. | 115.78 CH | 8 | 6.30, d, *J*=12.8 Hz | *E* |
| 14. | 114.94 CH | 3,5 | 6.80, d, *J*=6.8 Hz | *Z* |
| 15. | 64.67 $CH_2$ | 1’ | 4.19, t, *J*=5.2 Hz | *E* |
| 16. | 64.35 $CH_2$ | 1’ | 4.12,t, *J*=5.2 Hz | *Z* |
| 17. | 31.92 $CH_2$ | 16’ | 1.25-1.39,m | Both |
| 18. | 29.69* $CH_2$ | 6’,13’ | 1.25-1.39,m | Both |
| 19. | 29.65* $CH_2$ | 7’,12’ | 1.25-1.39,m | Both |
| 20. | 29.59* $CH_2$ | 8’,11’ | 1.25-1.39,m | Both |
| 21. | 29.54* $CH_2$ | 9’, 10’ | 1.25-1.39,m | Both |
| 22. | 29.36* $CH_2$ | 4’,15’ | 1.25-1.39,m | Both |
| 23. | 29.29* $CH_2$ | 5’,14’ | 1.25-1.39,m | Both |
| 24. | 28.76 $CH_2$ | 2’ | 1.69, quintet, *J*=5.6 Hz | Both |
| 25. | 25.98 $CH_2$ | 3’ | 1.25-1.39,m | *Z* |
| 26. | 25.97 $CH_2$ | 3’ | 1.25-1.39,m | *E* |
| 27. | 22.69 $CH_2$ | 17’ | 1.25-1.39,m | Both |
| 28. | 14.11 $CH_3$ | 18’ | 0.88, t, *J*=5.2 Hz | Both |
| 29. | OH (phenolic) | - | 5.41 | *E* |
| 30. | OH (phenolic) | - | 5.34 | *Z* |

*Assignments interchangeable

ethyl acetate (7:3) (Tables 2 and 3). This fraction was subjected to HPLC purification using dichloromethane as eluent and two main peaks (first with $t_R$=34 min and second $t_R$=48 min) were detected. Bioassay using *Cladosporium cucumerinum* revealed that fractions corresponding to these two peaks possessed antifungal activity. If the fraction were injected immediately after elution, HPLC showed that peak alone, but after exposure to daylight for a few hours, each of the peaks showed the presence of the other one indicating isomerization of the compounds. Since it was clearly known that the two isomers could not be separated under normal conditions, the compound corresponding to the major peak ($t_R$=48 min) was collected for further characterization. This purified fraction gave an approximate minimum inhibitory dose of 0.3 mg against the spore germination of *Cladosporium cucumerinum* on a TLC plate (Table 3). Antifungal activity of the purified fraction was further confirmed by the spore germination inhibition of *Alternaria alternata* and *A. porri* (Table 4). Attempts to obtain good quality crystals for X-ray diffraction studies failed. Powder diffraction also did not give any useful information.

Spectral characterization of the fraction having a m.p. 79-80°C (containing **1a** as the main constituent) was achieved using UV, IR, HRESIMS, CIMS, $^1$H NMR, $^{13}$C NMR, DEPT, COSY, HSQC and C, H analysis. The CIMS showed a $[M+H]^+$ ion at *m/z* 417, the HRESIMS a $[M+Na]^+$ ion at

**Table 2**: Antifungal activity of the extractives of *Ipomoea carnea* subsp. *fistulosa* against the mycelial growth of *Colletotrichum gloeosporioides*.

| Extractive/Fraction/Compound | Conc. | % mycelial growth inhibition |
|---|---|---|
| *n*-Hexane | 0.5% | 0.0 |
| Ethyl acetate | 0.5% | 24.2 (±0.8) |
| Methanol | 0.5% | 20.4 (±1.4) |
| Active fraction from column* | 0.5% | 68.5 (±0.7) |
| Phenol (standard) | 0.05% | 78.3 (±0.4) |

* Active fraction from column was obtained by elution with *n*-hexane-ethyl acetate (7:3) mixture

**Table 3**: Antifungal activity of the compounds of *Ipomoea carnea* subsp. *fistulosa* against *Cladosporium cucumerinum* by TLC bioautography.

| Extractive/Fraction/Compound | Dose (mg) | Inhibition** | MID |
|---|---|---|---|
| *n*-Hexane | 5.0 | - | ND |
| Ethyl acetate | 5.0 | + | ND |
| Methanol | 5.0 | ++ | ND |
| Active fraction from column* | 5.0 | +++ | ND |
| Octadecyl *p*-coumarate | 0.3 | + | 0.3 mg |
| Phenol (standard) | 0.3 | + | 0.3 mg |

* Active fraction from column was obtained by elution with *n*-hexane-ethyl acetate (7:3).

** The observations on TLC plate for *n*-hexane, ethyl acetate and methanol extractives and active column fraction* were made after elution with ethyl acetate ($R_f$ value of the inhibition spot ca 0.8). Observations on phenol and octadecyl *p*-coumarate were made by direct bioautography without elution after spotting the compounds quantitatively. ‘+’ indicates observable inhibition, ‘++’ indicates clear inhibition, ‘+++’ indicates very clear inhibition and ‘-’ indicates no inhibition. MID=minimum inhibitory dose; ND=Not determined.

*m/z* 439.3198, corresponding to a molecular weight of 416.3300, and C, H analysis gave C, 76.71%; H, 10.40%. These data gave the molecular formula as $C_{27}H_{44}O_3$ (required C, 77.8%; H, 10.45% and $M^+$ 416.3291). The $^1$H NMR spectrum showed two sets of closely related signal patterns indicating the presence of two isomers in the sample. The ratio of intensity of the peaks based on their coupling constants showed that the *E* and *Z* isomers exist in the ratio 2:1. This was also consistent with the 2:1 ratio of the areas of HPLC peaks with $t_R$=48 min and $t_R$=34. $^1$H-$^1$H COSY showed two sets of signal correlations for each of the isomers. In the first set, the signal at δ 7.62 (1H, d, *J*=12.8 Hz) correlated with that at δ 6.30, δ 7.43 (2H, d, *J*=6.8 Hz) with δ 6.84 and δ 4.19 (2H, t, *J*=5.2 Hz) with δ 1.69. In the second set, the signal at δ 7.63 (2H, d, *J*=6.8 Hz) correlated with that at δ 6.84, δ 6.83 (1H, d, *J*=10.0 Hz) with δ 5.83, and δ 4.12 (2H, t, *J*=5.2 Hz) with δ 1.63. $^1$H-$^{13}$C HSQC also showed two sets of correlations. In the first set, signal at $\delta_C$ 144.22 correlated with that at $\delta_H$ 7.62, $\delta_C$ 129.92 with $\delta_H$ 7.43, $\delta_C$ 115.84 with $\delta_H$ 6.84, $\delta_C$ 115.78 with $\delta_H$ 6.30 and $\delta_C$ 64.67 with $\delta_H$ 4.19. In the second set, signal at $\delta_C$ 143.18 correlated with that at $\delta_H$ 6.83, $\delta_C$ 132.30 with $\delta_H$ 7.63, $\delta_C$ 117.33 with $\delta_H$ 5.83, $\delta_C$ 114.94 with $\delta_H$ 6.80 and $\delta_C$ 64.35 with $\delta_H$ 4.12. The $^{13}$C NMR spectrum showed 28 signals, which were assigned for both *E/Z* isomers taking into consideration DEPT, COSY and HSQC data (Table 1). All these results led to the conclusion that the active fraction is a mixture of (*E*)-octadecyl *p*-coumarate (**1a**) and (*Z*)-octadecyl *p*-coumarate (**1b**).

The isomerization of (*E*)-octadecyl *p*-coumarate and (*Z*)-octadecyl *p*-coumarate is shown in Figure 1. Our finding of the isomerization of octadecyl *p*-coumarate corroborates

the reports on the isomerization of structurally related eicosanyl *p*-coumarates isolated from *Psiadia punctulata* [11] and 21'-hydroxyheneicosanyl-4-hydroxy-(*cis*- and *trans*) *p*-coumarate isolated from *Tanacetum longifolium* [12].

The fact that four earlier reports [9b-9e] on the isolation of octadecyl *p*-coumarates were from the genus *Ipomoea* may be of chemotaxonomic interest.

## Experimental

***General:*** UV spectra were obtained with a Spectronic UV-Visible spectrophotometer. IR spectra were obtained on a Perkin-Elmer spectrophotometer. $^{1}$H NMR and $^{13}$C NMR spectra were recorded on a Bruker spectrometer operating at 400 MHz and 100 MHz, respectively (Table 1). HRESIMS were obtained on a Micromass Q-TOF apparatus. Carbon-Hydrogen-Nitrogen analysis was obtained using a ThermoFinnigan Flash EA 1112 CHNS analyzer. HPLC purification was achieved using a Waters HPLC system (515 pump, 7725 Rheodyne injector, Waters 2487 Dual λ absorbance detector) under conditions as follows: Column Prep Nova Pak HR Silica 7.8 x 300 mm, flow rate 1 mL/min, UV detection at 254 nm, eluent $CH_2Cl_2$. TLC bioassay was achieved with silica gel plates (0.5 mm thickness). Observation of spore germination inhibition was made using a Carl Zeiss Axio Imager AI microscope.

***Plant material:*** The leaves of *Ipomoea carnea* subsp. *fistulosa* were collected from the farm of the Indian Institute of Horticultural Research, Hessaraghatta Lake P.O., Bangalore – 560089, India and a voucher specimen is kept at the Section of Medicinal Crops of the Institute.

***Extraction and isolation:*** The dried plant material (2 Kg) was extracted first with *n*-hexane, then with ethyl acetate and finally with methanol. Column chromatography of the ethyl acetate extractive utilizing silica gel with *n*-hexane-ethyl acetate mixtures with increasing percentages of ethyl acetate and TLC bioassay were conducted side by side. The fraction which showed maximum activity was taken for HPLC purification. Two main peaks ($t_R$=34 min and $t_R$=48 min) were detected and the eluents corresponding to these peaks were collected separately for further investigation.

### *Antifungal activity assays*

***Poisoned food technique:*** Pure culture of *Colletotrichum gloeosporioides* ITCC 4573 obtained from Indian Type Culture Collections, Indian Agricultural Research Institute, New Delhi, India was used for this study [13]. Percent mycelial growth inhibition values presented in Table 2 are the averages of 2 replications, standard deviation being presented in parenthesis. The purified HPLC fraction was not used in this technique because of the paucity of material and its poor solubility both in water and solvents miscible with water.

**Table 4**: Spore germination inhibition of *Alternaria alternata and A. porri* by octadecyl *p*-coumarates (*E* and *Z* isomers in ratio 2:1).

| Treatment | *Alternaria alternata* | | *Alternaria porri* | |
|---|---|---|---|---|
| | % germination | % inhibition w.r.t. control | % germination | % inhibition w.r.t. control |
| Control (3% *n*-propanol in water) | 92.0 (±0.5) | - | 95.0 (±0.0) | - |
| Octadecyl coumarates (100 mg/L) | 67.0 (±1.4) | 26.6 (±0.8) | 71.5 (±0.7) | 24.8 (±0.8) |
| Octadecyl coumarates (500 mg/L) | 51.5 (±2.1) | 44.1 (±2.3) | 55.0 (±2.8) | 42.1 (±3.0) |
| Phenol (standard) (100 mg/L) | 86.0 (±0.0) | 6.5 (±0.0) | 91.0 (±1.4) | 4.3 (±1.5) |
| Phenol (standard) (500 mg/L) | 71.5 (±0.7) | 22.3 (±0.8) | 79.0 (±2.8) | 16.8 (±3.0) |

***TLC bioautography:*** A pure culture of *Cladosporium cucumerinum* IMI 249540 obtained from the International Mycological Institute, U.K., maintained on a potato-dextrose-agar (PDA) medium was used for this assay [14].

***Spore germination inhibition study:*** For this study [13], spores of *Alternaria alternata* from infected tomato fruits and *A. porri* from infected onion leaves collected from the IIHR experimental farm in Hessaraghatta, Bangalore, India were used. Spores were added to a solution of the compound in 3% *n*-propanol in water kept in cavity slides by the hanging drop method. Observation on spore germination was recorded after incubation for 3 h. Percent spore germination inhibition values presented in Table 4 are the averages of 2 replications, standard deviations being given in parenthesis.

**(*E*)-Octadecyl *p*-coumarate (1a):** It was collected at $t_R$ of 48 min. as major peak during HPLC separation. The compound got partly converted to (*Z*)-octadecyl *p*-coumarate after a few hours. White solid with a faint yellowish to greenish tinge (20 mg).
MP: 79-80ºC.
IR: 3393 (OH stretching), 2921(C-H stretching), 2880 (C-H stretching), 1713 (α, β unsaturated ester), 1674 (C=C of phenol), 1604 (C=C of α, β unsaturation), 1586 (aromatic C=C), 1516 (aromatic C=C), 1468 (C-H), 1377 ($CH_3$), 1307 (C-O stretching ) 1274 (C-O stretching), 1170 (C-O stretching), 982 (C=C conjugated to C=O), 835 (C=C-H), 722 ($CH_2$), 517 cm$^{-1}$.
UV (MeOH) $\lambda_{max}$: 225, 308 nm. Second peak showed bathochromic shift on addition of NaOH.
$^{1}$H NMR and $^{13}$C NMR ($CDCl_3$): Table 1.
CIMS: 417 $[M+H]^+$, 164 (HO-$C_6H_4$ CH=COOH) 147 (HO-$C_6H_4$CH=CO), 129,120,107.
HRESIMS $[M+Na]^+$ 439.3198 (required for $C_{27}H_{44}O_3Na$ 439.3189).

Elemental analysis: Found C, 76.71; H, 10.40 ($C_{27}H_{44}O_3$ requires C, 77.80; H, 10.45).

Direct bioautography on TLC plate was done using *Cladosporium cucumerinum* (Table 3). The activity was further confirmed by spore germination inhibition of *Alternaria alternata* and A. *porri* (Table 4).

**(*Z*)-Octadecyl *p*-coumarate (1b):** It was collected at $t_R$ of 34 min as minor peak during HPLC separation. It got partly converted to *E*-octadecyl *p*-coumarate after a few hours. White solid with a yellowish to greenish tinge (10 mg).
MP: 79-80ºC.
$^1$H NMR and $^{13}$C NMR ($CDCl_3$): Table 1.

**Acknowledgements -** The authors thank Dr A.S. Sidhu, Director, I. I. H. R. for encouragement. They are grateful to NMR Research Centre, Indian Institute of Science, Bangalore, India for spectral data.

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# 2011
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