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Rapid preparation of (methyl)malonyl coenzyme A and enzymatic formation of unusual polyketides by type III polyketide synthase from *Aquilaria sinensis*

Bo-Wen Gao [a,b,c], Xiao-Hui Wang [a], Xiao Liu [a], She-Po Shi [a,*], Peng-Fei Tu [a,*]

[a] Modern Research Center for Traditional Chinese Medicine, Beijing University of Chinese Medicine, Beijing 100029, China
[b] School of Chinese Materia Medica, Beijing University of Chinese Medicine, Beijing 100102, China
[c] Baotou Medical College, Baotou 014060, China

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ABSTRACT

(Methyl)malonyl coenzyme A was rapidly and effectively synthesized by a two-step procedure involving preparation of *N*-hydroxysuccinimidyl (methyl)malonate from (methyl)Meldrum's acid, and followed by transesterification with coenzyme A. The synthesized (methyl)malonyl coenzyme A could be well accepted and assembled to 4-hydroxy phenylpropionyl coenzyme A by type III polyketide synthase from *Aquilaria sinensis* to produce dihydrochalcone and 4-hydroxy-3,5-dimethyl-6-(4-hydroxyphenethyl)-*2H*-pyrone as well as 4-hydroxy-3,5-dimethyl-6-(5-(4-hydroxyphenyl)-3-oxopentan-2-yl)-*2H*-pyrone.

Malonyl coenzyme A (malonyl-CoA) and methylmalonyl coenzyme A (methylmalonyl-CoA) are universally used as chain extenders by polyketide synthases (PKSs) to synthesize a large number of structurally diverse polyketides[1–10] exhibiting a vast array of biological and pharmacological activities such as antibacterial,[11,12] antifungal,[13,14] anticancer,[15] antioxidation,[16] and immunosuppressive properties.[17] As the most common chain extenders, (methyl)malonyl-CoA could be repeatedly assembled to a start coenzyme A thioester by PKSs via decarboxylative Claisen condensation to construct a linear poly-β-ketone intermediate which could finally format structurally varied polyketides by sequential modifications such as reduction, cyclization and aromatization.[18–20] Owing to the wide range of substrate tolerance and unordinary flexibility of structural modification, PKSs are ideal biocatalysts for construction of unusual molecule libraries for screening of new drug candidates,[21,22] while the high cost of commercially available (methyl)malonyl-CoA prohibits large scale in vitro synthesis of these unusual molecules. In previous reports, enzymes related to the formation of malonyl-CoA such as acetyl-CoA carboxylase[23] and malonyl-CoA synthase[24–27] have been cloned and applied to enzymatically synthesize malonyl-CoA. However, enzyme catalyzed synthesis of malonyl-CoA involves laborious gene cloning and protein preparation, and always accompanying with low yield of malonyl-CoA. Besides of enzymatic synthesis of malonyl-CoA, chemical synthesis of (methyl)malonyl-CoA by transesterification of (methyl)malonic monothiophenyl ester[28–30] or *S*-malonyl-*N*-decanoyl cysteamine[31] with Coenzyme A have also been reported. However, these methods are hard to differentiate the two ends of (methyl)malonic acid, which results in the preparation of monothiophenyl ester or *S*-malonyl-*N*-decanoyl cysteamine nonselective, and multiple steps for purification of the committed intermediate are needed. It has been reported that malonyl-CoA could be directly synthesized by stirring coenzyme A in cold malonyl dichloride,[32] while the low yield of malonyl-CoA made this method not practically useful to prepare large amount of malonyl-CoA. Therefore, it is still a challenge to rapidly and cost effectively synthesize large amount of (methyl)malonyl-CoA.

Here we report a more rapid and cost effective method to synthesize (methyl)malonyl-CoA with high yield (more than 90% according to the amount of coenzyme A used). The two-step procedure involves preparation of *N*-hydroxysuccinimidyl (methyl)malonate via *N*-hydroxysuccinimide nucleophilic attacking commercially available (methyl)Meldrum's acid, and followed by transesterification with coenzyme A. Notably, the purity of the synthesized (methyl)malonyl-CoA could reach to more than 90% after purification by a simple procedure using Sephadex LH-20 column chromatography. Enzymatic reaction demonstrated

* Corresponding authors. Tel./fax: +86 10 64286350.
E-mail addresses: shishepo@163.com (S.-P. Shi), pengfeitu@163.com (P.-F. Tu).

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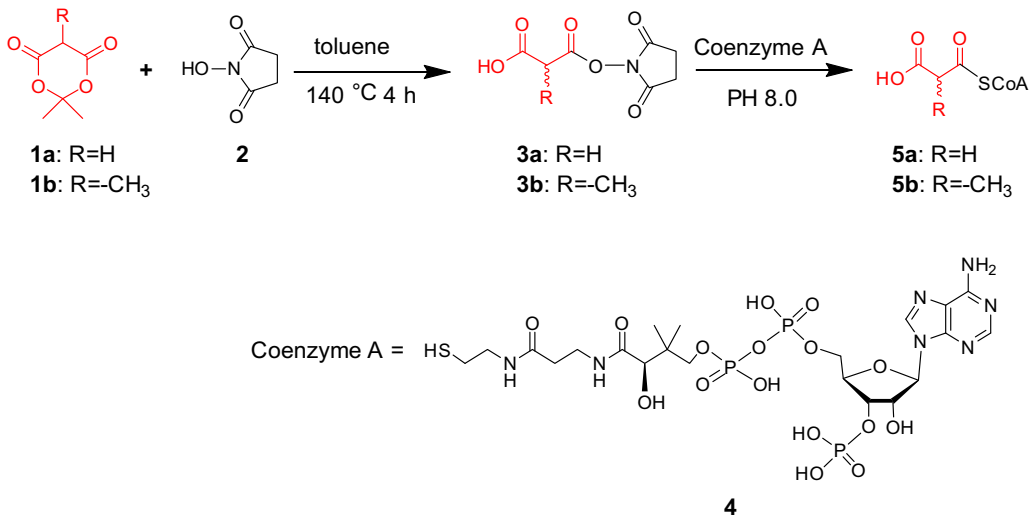
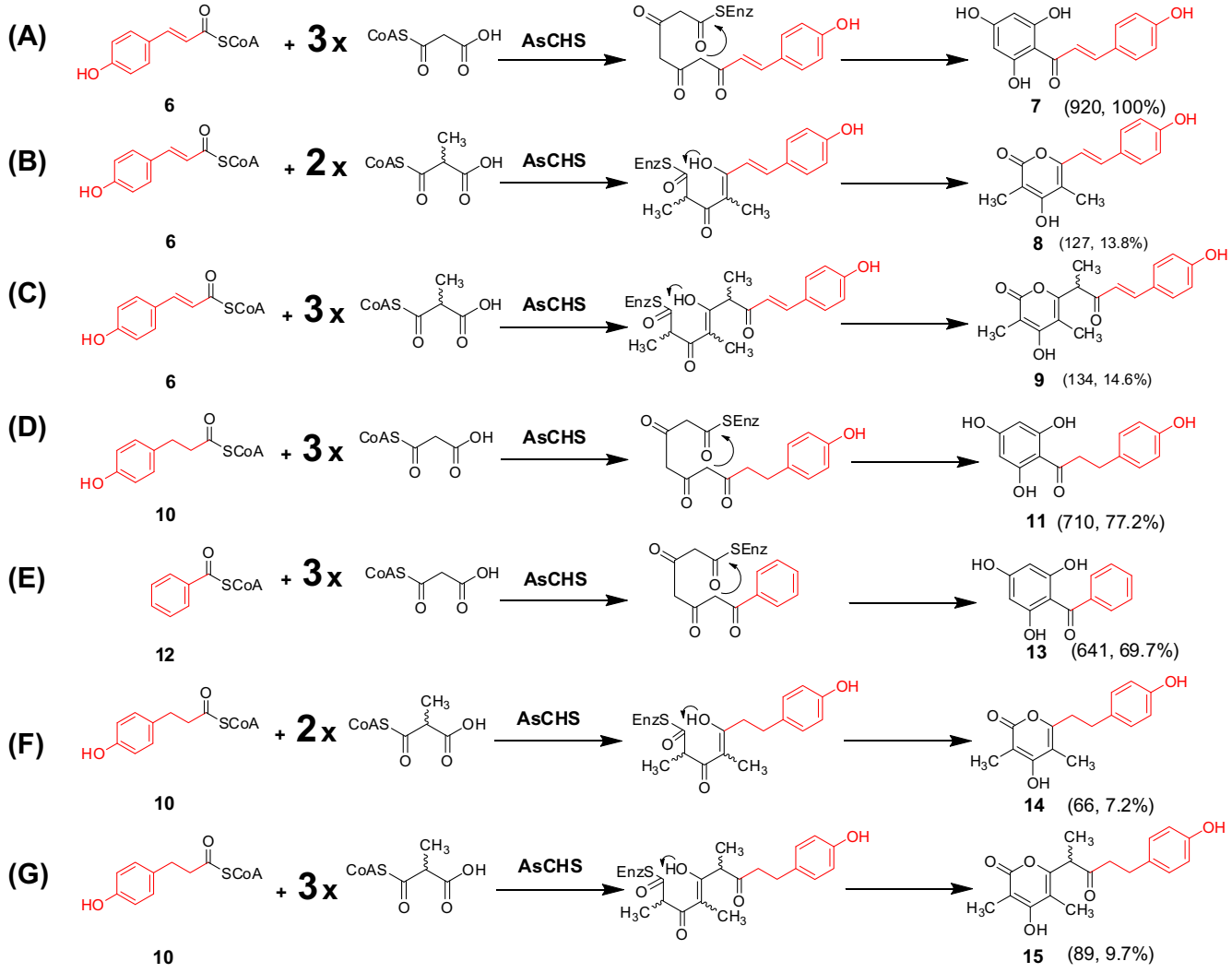


Scheme 1. Strategy for (methyl)malonyl-CoA synthesis.



Scheme 2. Proposed mechanism for enzymatic formation of varied polyketides catalyzed by AsCHS. The numbers in each parenthese are the values of absolute peak area of each enzymatic product in HPLC and percentage relative to the maximum peak area of naringenin chalcone (**7**).

that (methyl)malonyl-CoA prepared by the present method could be well accepted by type III PKS from *Aquilaria sinensis* to produce structurally unusual polyketides such as dihydrochalcone from the condensation of 4-hydroxy phenylpropionyl-CoA with three molecules of prepared malonyl-CoA, and 4-hydroxy-3,5-dimethyl-6-(4-hydroxyphenethyl)-*2H*-pyrone from the condensation of 4-hydroxy phenylpropionyl-CoA with two molecules of prepared methylmalonyl-CoA as well as 4-hydroxy-3,5-dimethyl-6-(5-(4-hydroxyphenyl)-3-oxopentan-2-yl)-*2H*-pyrone from the condensation of 4-hydroxy phenylpropionyl-CoA with three molecules of prepared methylmalonyl-CoA.

Meldrum's acid (**1a**) and *N*-hydroxysuccinimide (**2**) were refluxed in toluene for 4 h, the reaction mixture was completely removed the solvent under reduce pressure, and the residue was recrystallized in absolute ethanol to afford the key intermediate *N*-hydroxysuccinimidyl malonate (**3a**). The purified intermediate, *N*-hydroxysuccinimidyl malonate, was subsequently stirred with coenzyme A (**4**) in weak alkaline aqueous solution (pH = 8.0) to yield malonyl-CoA (**5a**) (Scheme 1). The reaction progress was monitored by HPLC showing the appearance of malonyl-CoA accompanied by the disappearance of coenzyme A. The reaction was terminated when the free coenzyme A completely disappeared after 12 h later, and the reaction mixture was purified by Sephadex LH-20 column chromatography to afford malonyl-CoA. HPLC analysis suggested that excess *N*-hydroxysuccinimidyl malonate in the reaction mixture could be completely removed by Sephadex LH-20 column chromatography, and the purity of malonyl-CoA reached to 95%. The purified malonyl-CoA was identified by comparing its retention time in the HPLC chromatogram and UV spectrum to those of the authentic malonyl-CoA. The retention time of synthetic malonyl-CoA in HPLC chromatogram was completely identical to that of the authentic malonyl-CoA. Additionally, the UV spectrum of the synthesized malonyl-CoA indicated superposable feature to that of the authentic malonyl-CoA. ESI-MS spectrum showed the presence of three quasimolecular ion peaks at *m*/*z* 854 $[M+H]^+$, 876 $[M+Na]^+$, and 892 $[M+K]^+$ which provided further powerful evidence for the identification of the synthetic malonyl-CoA (Supplementary data).

As a commercially available and commonly used substrate for acylation chemistry,[33–35] Meldrum's acid could specifically differentiate the two ends of malonic acid, and thus leading to significantly increase the yield of the key intermediate *N*-hydroxysuccinimidyl malonate. Furthermore, the moderate reaction conditions and the convenient purification process avoided the unexpected loss and hydrolysis of malonyl-CoA.

Using the same strategy, methylmalonyl-CoA could also be synthesized from methylMeldrum's acid (**1b**) (Scheme 1). In the ESI-MS spectrum of the synthesized methylmalonyl-CoA (**5b**), quasimolecular ion peaks at *m*/*z* 868 $[M+H]^+$ and 978 $[M+5Na-4H]^+$ were observed which was identical to the data reported by Padmakumar.[30] Comparing the NMR data of the synthesized methylmalonyl-CoA with those of the free coenzyme A, a methyl group presented at δ 1.37 (d, J = 7.6 Hz) and the sulfureted methylene group presented at relative downfield δ 3.11 (t, J = 6.2 Hz) in the

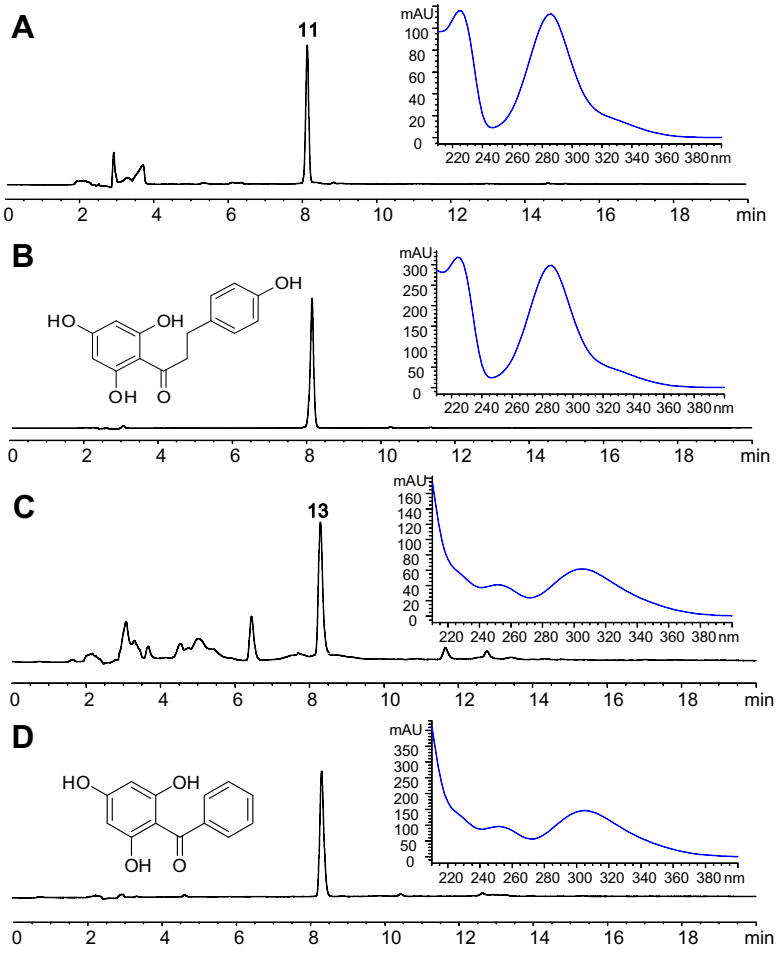


Figure 1. The HPLC elution profiles and UV spectra of AsCHS enzyme reaction products and the authentic compounds. The enzyme product from malonyl-CoA and 4-hydroxy phenylpropionyl-CoA (A), the authentic compound of phloretin (B), the enzyme product from malonyl-CoA and benzoyl-CoA (C), and the authentic compound of 2,4,6-trihydroxybenzophenone (D).

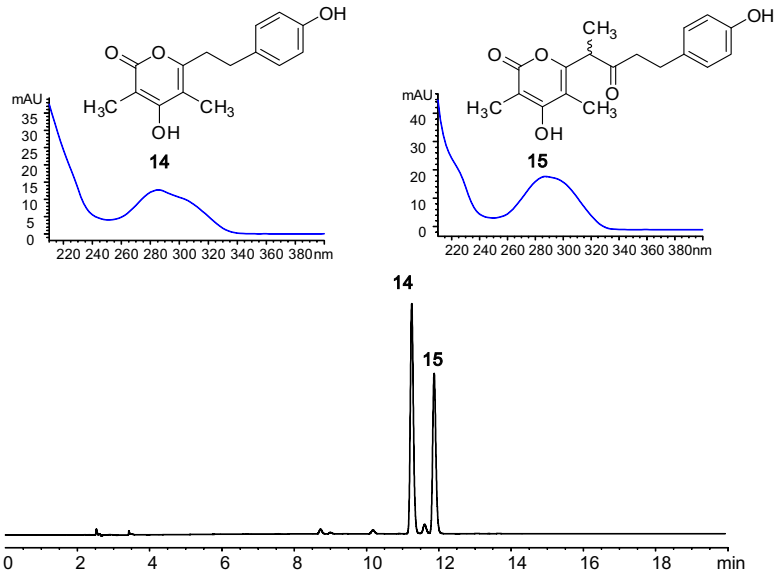


Figure 2. The HPLC elution profile and UV spectra of the AsCHS enzyme reaction products **14** and **15** from 4-hydroxy phenylpropionyl-CoA and methylmalonyl-CoA.

^{1}H NMR spectrum as well as the signals presented at δ 14.3 and 201.8 in the ^{13}C NMR spectrum of the synthesized methylmalonyl-CoA suggested that a methylmalonic acid moiety was successfully introduced into the molecular by transesterification[36] (Supplementary data).

In order to test the activity of the synthesized (methyl)malonyl-CoA, a type III polyketide synthase (AsCHS) was cloned from *Aquilaria sinensis*. AsCHS shares 77.4% amino acid sequences identity to those of chalcone synthase from *Medicago sativa*[37] and the highly conserved Cys-His-Asn cytalytic triad of type III polyketide synthase could also be observed in AsCHS. Phylogenetic analysis revealed that AsCHS grouped with other chalcone-producing type III polyketide synthases, including *Medicago sativa* CHS2, *Rheum palmatum* CHS2, *Scutellaria baicalensis* CHS, and *Ruta graveolensis* CHS (Supplementary data). The Chalcone-producing function of AsCHS was confirmed by enzymatic formation of naringenin chalcone (**7**) from the condensation of 4-coumaroyl-CoA (**6**) with three molecules of malonyl-CoA. Additionally, when methylmalonyl-CoA was used as the chain extender, AsCHS could catalyze the condensation of 4-coumaroyl-CoA with two or three molecules of methylmalonyl-CoA to produce two known polyketides, (*E*)-4-hydroxy-6-(4-hydroxystyryl)-3,5-dimethyl-*2H*-pyrone (**8**) and (*E*)-4-hydroxy-6-(5-(4-hydroxyphenyl)-3-oxopent-4-en-2-yl)-3,5-dimethyl-*2H*-pyrone (**9**), respectively (Scheme 2).[38]

When AsCHS was incubated with the synthesized malonyl-CoA and 4-hydroxy phenylpropionyl-CoA (**10**), a dihydrochalcone with molecular weight *m*/*z* 274 was produced. The structure of the dihydrochalcone was unambiguously identified as phloretin (**11**) by analyzing its ESI-MS data and comparing with authentic compound using HPLC (Fig. 1A and B) (Scheme 2). Phloretin, as a key intermediate of the biosynthetic pathway of flavonoids in plants, has not been isolated from *A. Sinensis*, but commonly occurred in the peel of juicy fruit such as apple and strawberries with remarkably antioxidant activity.[16] On the other hand, AsCHS could catalyze the formation of 2,4,6-trihydroxybenzophenone (**13**) from the condensation of benzoyl-CoA (**12**) and three molecules of malonyl-CoA (Scheme 2) (Fig. 1C and D). Notably, benzophenone and its glycosides such as iriflophenone 3-*C*-β-D-glucoside and iriflophenone 2-*O*-α-L-rhamnoside have been already isolated from the leaves of *A. Sinensis*.[39]

Remarkably, When AsCHS was incubated with the synthesized methylmalonyl-CoA and 4-hydroxy phenylpropionyl-CoA, the enzymatic reaction generated a 1.3:1 mixture of two products having different parent ion peaks at *m*/*z* 261.1114 $[M+H]^+$ and 317.1379 $[M+H]^+$ in the LCMS-IT-TOF spectra (Fig. 2). The ^{1}H NMR spectrum of **14** showed the presence of two methyl singlets at δ 1.90 (3H, s) and 1.72 (3H, s), two methylene triplets at δ 2.85 (2H, t, J = 6.8 Hz) and 2.78 (2H, t, J = 6.8 Hz), and four aromatic protons at δ 6.96 (2H, d, J = 8,4 Hz,) and 6.67 (2H, d, J = 8.4 Hz), respectively. The ^{13}C NMR spectrum of **14** showed the presence of two methyl carbons at δ 8.5 and 7.5, carbons attributed to 4-hydroxy phenylethyl group at δ 155.4, 131.0, 129.0, 114.7, 32.6, and 32.2, and the presence of carbons due to lactone ring at δ 168.1, 167.4, 157.4, 109.3 and 97.1, respectively. In the NMR spectra of **15**, the signals due to 4-hydroxy phenylethyl moiety and 3,5-dimethyl-4-hydroxy lactone moiety were typically observed. In addition, a methyl doublet at δ 1.31 (3H, d, J = 6.7 Hz), and a methine multiplet at δ 3.88 (1H, m) in the ^{1}H NMR spectrum of **15**, and a methyl carbon at δ 12.0, a methine carbon at δ 48.3, and a carbonyl carbon at δ 207.0 in the ^{13}C NMR spectrum of **15** were presented. By analysis of the spectroscopic data including ^{1}H, ^{13}C, HSQC, and HMBC, the structures of the new polyketides **14** and **15** were unambiguously elucidated as 4-hydroxy-3,5-dimethyl-6-(4-hydroxyphenethyl)-*2H*-pyrone **14** and 4-hydroxy-3,5-dimethyl-6-(5-(4-hydroxyphenyl)-3-oxopentan-2-yl)-*2H*-pyrone **15**, respectively[40,41] (Scheme 2) (Supplementary data).

In summary, the present report provides a rapid and cost effective method to synthesize large amount of (methyl)malonyl-CoA, and exemplary synthesized varied polyketides by a type III PKS from *Aquilaria sinensis*, which could be meaningful and luminous for in vitro construction of unusual molecule libraries using PKSs.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2015.01.045.

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36. *Methylmalonyl-CoA:* HPLC-UV: R_t = 7.1 min, λ_{max} 259 nm. ESI-MS (positive): 868 $[M+H]^+$, 978 $[M+5Na-4H]^+$. ^1H NMR (500 MHz, D_2O): δ 8.61(1H, s), 8.28 (1H, s), 6.25 (1H, d, J = 6.2 Hz), 4.94 (2H, t, J = 6.8 Hz), 4.36 (2H, s), 4.12 (1H, s), 3.94 (1H, d, J = 6.2 Hz), 3.67 (2H, d, J = 6.2 Hz), 3.55 (2H, d, J = 6.2 Hz), 3.44 (2H, d, J = 6.2 Hz), 3.11 (2H, d, J = 6.2), 2.54 (2H, t, J = 6.5 Hz), 1.37 (3H, d, J = 7.6 Hz), 0.99 (3H, s), 0.86 (3H, s). ^{13}C NMR (125 MHz, D_2O): δ 201.8, 176.9, 174.6, 173.8, 155.2, 152.5, 149.0, 139.8, 118.3, 86.5, 83.5, 74.1, 73.9, 71.8, 65.4, 38.5, 38.3, 38.2, 35.4, 35.2, 28.0, 20.8, 18.1, 14.3.
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40. *4-Hydroxy-3,5-dimethyl-6-(4-hydroxyphenethyl)-2H-pyrone* (***14***)*:* HPLC-UV: R_t = 11.2 min, λ_{max} 297 nm. HRESIMS (positive): found for $[C_{15}H_{16}O_4]^+$ 261.1114; calcd 261.1121. ^1H NMR (500 MHz, CD_3OD): δ 6.96 (2H, d, J = 8,4 Hz), 6.67 (2H, d, J = 8.4 Hz), 2.85 (2H, t, J = 6.8 Hz), 2.78 (2H, t, J = 6.8 Hz), 1.90 (3H, s), 1.72 (3H, s). ^{13}C NMR (125 MHz, CD_3OD): δ 168.1, 167.4, 157.4, 155.4, 131.0, 129.0, 114.7, 109.3, 97.1, 32.6, 32.2, 8.5, 7.5.
41. *4-Hydroxy-3,5-dimethyl-6-(5-(4-hydroxyphenyl)-3-oxopentan-2-yl)-2H-pyrone* (***15***)*:* HPLC-UV: R_t = 11.9 min, λ_{max} 297 nm. HRESIMS (positive): found for $[C_{18}H_{20}O_5]^+$ 317.1379; calcd 317.1384. ^1H NMR (500 MHz, CD_3OD): δ 6.92 (2H, d, J = 8,4 Hz), 6.43 (2H, d, J = 8.4 Hz), 3.88(1H, m), 2.71 (4H, m), 1.94 (3H, s), 1.90 (3H, s), 1.31 (3H, d, J = 6.7 Hz). ^{13}C NMR (125 MHz, CD_3OD): δ 207.0, 169.7, 167.0, 155.2, 154.9, 131.5, 128.8, 114.7, 111.4, 97.6, 48.3, 42.0, 28.7, 12.0, 8.9, 7.6.