Journal of Medicinal Chemistry

ARTICLE

pubs.acs.org/jmc

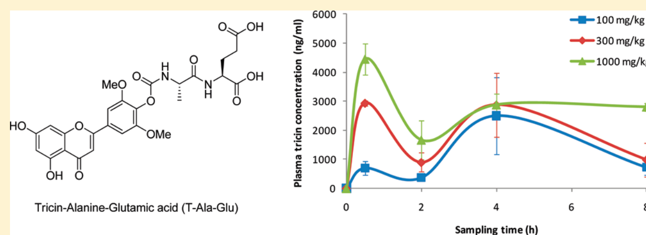
Increased Bioavailability of Tricin–Amino Acid Derivatives via a Prodrug Approach

Masayuki Ninomiya,[†] Kaori Tanaka,[§,||] Yuzo Tsuchida,[⊥] Yoshinori Muto,[‡] Mamoru Koketsu,[*,†] and Kunitomo Watanabe[*,§,||]

[†]Department of Materials Science and Technology, Faculty of Engineering, [‡]Department of Functional Bioscience, School of Medicine, [§]Division of Anaerobe Research, Life Science Research Center, and [||]United Graduate School of Drug Discovery and Medical Information Sciences, Gifu University, 1-1 Yanagido, Gifu 501-1194, Japan

[⊥]Hououdou Co., Ltd., 4-3-2 Ebara, Shinagawa, Tokyo 142-0063 Japan

ABSTRACT: Tricin (4′,5,7-trihydroxy-3′,5′-dimethoxyflavone) has demonstrated diverse biological activities. This compound has a high anti-human cytomegalovirus (HCMV) activity; however, its oral availability is low. To improve its bioavailability, we synthesized tricin–amino acid derivatives as prodrugs and investigated their cell permeability, stability in vitro, and oral availability in vivo. The results demonstrated that the tricin–alanine–glutamic acid conjugate exhibited enhanced permeability, stability in MDCK cells, and excellent bioavailability after oral administration in Crl:CD (SD) male rats. Tricin–alanine–glutamic acid conjugate is a potential new anti-HCMV drug.



INTRODUCTION

Flavonoids constitute a large family of natural products that can be found in nearly all plant species. At present, flavonoids comprise more than 6500 natural compounds.[1] The study of flavonoids is becoming increasingly interesting because of their important metabolic roles in plants and animals and their potent and diversified biological activities.[2] In particular, flavones, a subclass of flavonoids, are of interest due to having biological activities such as inhibition of retroviral reverse transcriptases,[3,4] protein–tyrosine kinases,[5,6] and serine–threonine kinases.[5] They possess anticancer[7,8] and chemopreventive effects[8,9] and inhibit HIV-induced syncytium formation.[10]

The flavone tricin (4′,5,7-trihydroxy-3′,5′-dimethoxyflavone)[11] occurs in its glycoside form in Poaceae (formerly Gramineae) plants including rice, oats, barley, and wheat.[12,13] This compound has previously been reported to possess antioxidant activity,[14] assessed using the 2,2′-azobis(2,4-dimethylvaleronitrile) (AMVN)-initiated oxidation assay system with methyl linolate.[15] It has been reported that tricin shows antiviral,[16] anti-inflammatory,[17] antihistamic,[18] and anticancer activities.[19] Its role in cancer prevention is also an active area of research.[20,21]

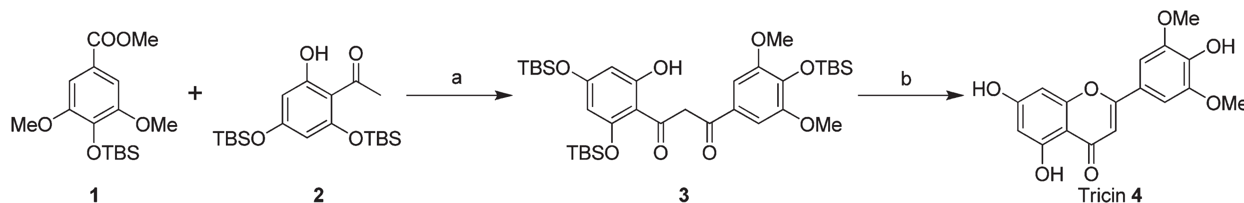
Recently, our phytochemical research led to the isolation of tricin from a hot water extract of *Sasa albo-marginata* leaves. The isolated tricin showed anti-human cytomegalovirus (HCMV) activity in studies of the replication of HCMV in MRC-5 cells.[22] Tricin exhibited a distinct anti-HCMV effect with an EC_{50} of 0.17 $\mu g\ mL^{-1}$; in contrast, ganciclovir (GCV), which is an anti-HCMV drug in the marketplace, had an EC_{50} of 1.03 $\mu g\ mL^{-1}$.[23] This result demonstrates that the antiviral activity of tricin is stronger than that of GCV. Tricin greatly reduced production of HCMV virions in MRC-5 cells but did not inhibit growth of the host cells. Thus, tricin was determined to be sufficiently safe for clinical development as a candidate anti-HCMV drug.

In this study, we first prepared a large quantity of tricin and evaluated its bioavailability after oral administration in Crl:CD (SD) rats in vivo; however, the plasma tricin concentration was quite low, as is typically the case for other flavonoids. To date, the clinical use of flavonoids has been limited by their poor bioavailability. The bioavailability of tricin is similar to that of quercetin, which also has a wide range of biological activities.[24] To overcome this limitation, a variety of modifications of quercetin, such as glycosylation,[25] acylation,[26] and liposomalization,[27] have been performed. Similarly, Cai et al. recently attempted to improve the oral bioavailability of tricin via a methylation approach.[28] In addition, Kim et al. reported that quercetin–amino acid derivatives demonstrate favorable cell permeability in vitro through interaction with the peptide transporter PepT1.[29] In the present study, we endeavored to improve the oral bioavailability of tricin via a prodrug approach. To that end, we synthesized several amino acid derivatives of tricin as prodrugs and evaluated the relative cell permeabilities and half-lives of the tricin–amino acid derivatives in Madin–Darby canine kidney (MDCK) cells in vitro and associated plasma tricin concentrations in male rats in vivo. The results demonstrated that the tricin–alanine–glutamic acid conjugate (T-Ala-Glu) exhibited excellent bioavailability after oral administration, which was clearly confirmed by an increase in cellular permeability.

Received: December 3, 2010
Published: February 14, 2011

ACS Publications © 2011 American Chemical Society

Scheme 1. Synthesis of Tricin 4[a]



[a] Reagents and conditions: (a) LiHMDS, THF, −78 °C → room temperature, 3 days. (b) 0.5% H_2SO_4 in AcOH, 100 °C, overnight.

RESULTS AND DISCUSSION

Preparation of Tricin. Previously, we isolated tricin from *S. albo-marginata* and confirmed its anti-HCMV activity.[22,30] In this study, we used a chemical synthesis approach because tricin was required in gram quantities. We prepared tricin through a condensation reaction of methyl benzoate **1** with acetophenone **2**, followed by acid cyclodehydration.[31] The required methyl 4-*O*-*tert*-butyldimethylsilyl-3,5-dimethoxybenzoate **1** and 2′,4′-*O*-bis(*tert*-butyldimethylsilyl)-6′-hydroxyacetophenone **2** were prepared by protection of suitable starting materials with *tert*-butyldimethylsilyl chloride in tetrahydrofuran (THF) in the presence of *N*,*N*-diisopropylethylamine (DIPEA). The condensation reaction of **1** with 8 equiv of lithium bis(trimethylsilyl)amide (LiHMDS) and 1.5 equiv of **2** in THF at −78 °C raised to room temperature over 3 days gave intermediate **3** as a mixture of tautomers. These were subjected to acid cyclodehydration and deprotection with 0.5% H_2SO_4 in acetic acid at 100 °C overnight. These reaction conditions afforded the corresponding tricin **4** at an overall yield of 68% (105.0 g). The purity of the tricin was checked using reverse-phase high-performance liquid chromatography (RP-HPLC) and was confirmed to be 99% (Scheme 1).

Plasma Tricin Concentration after Oral Administration in Rats. Tricin was orally administered to male and female Crl:CD (SD) rats at doses of 100, 300, and 1000 mg kg^{-1}. Blood was collected 0, 0.5, 2, and 4 h after administration of tricin. Quantitation of tricin in plasma was conducted by RP-HPLC.[32]

For male rats, a small amount of tricin was detected in the plasma 0.5, 2, and 4 h after tricin administration (Figure 1). In the 300 and 1000 mg kg^{-1} groups, the plasma tricin concentrations after 2 and 4 h were nearly the same. The results for female rats were different from those for male rats. In the 100 and 300 mg kg^{-1} groups of females, tricin was not detected in the plasma after 0.5, 2, or 4 h. In addition, in the 1000 mg kg^{-1} group, tricin was detected in the plasma after administration but gradually decreased after peaking at 0.5 h. Collectively, our in vivo measurements indicated that plasma tricin concentrations at all dosages in both males and females were quite low, less than 60 ng mL^{-1}.

To date, tricin has been investigated as a putative cancer chemopreventive agent, and Cai et al. performed an experiment in which it was administered orally to C57BL/6J mice.[33] They also reported low concentrations of orally administered tricin in the mouse plasma. It is generally assumed that tricin is poorly absorbed from the gastrointestinal tract (GIT) in its native form. A substantial portion of tricin is metabolized by UDP-glucuronosyltransferases[34] in the gut mucosal cells, and approximately half of the glucuronidates are excreted back into the gut lumen, mainly through the multidrug resistance protein multidrug resistance protein 2 (MRP2) pump.[35,36] The unmetabolized tricin is then extensively metabolized in the liver and excreted via bile.[37] It has also been reported that liver microsomes from immature and adult female rats catalyzed glucuronidation of estrone and estradiol more rapidly than did liver microsomes from age-matched male rats.[38] Thus, the differing results for the male and female groups shown in Figure 1 may be due to immediate metabolism of tricin, similar to estrogens, in the female groups but not in the male groups. The results demonstrated that tricin was not orally available in its native form. Therefore, modifications were necessary to improve tricin bioavailability.

Synthesis of Tricin Prodrugs. A significant problem in tricin oral delivery is its low bioavailability after oral administration, as demonstrated by our data. As discussed above, poor absorption and rapid depredation may contribute to this problem. Therefore, it is necessary to increase the absorption rate of tricin as well to decrease the rate of metabolism. Previously, QC12 [3′-(*N*-carboxymethyl)carbamoyl-3,4′,5,7-tetra-hydroxyflavone; quercetin 3′-glycine conjugate], a quercetin prodrug, had been designed and evaluated for preclinical and clinical pharmacokinetic properties.[39] Several quercetin−amino acid derivatives were prepared, and their solubility, stability, and permeability in vitro were estimated.[29] The results of these studies suggested that conjugation with amino acids was the most promising approach. To identify a tricin prodrug with improved drug availability, we synthesized amino acid derivatives from tricin.

A series of tricin−amino acid conjugates were synthesized. Six amino acids, including four neutral amino acids (glycine, Gly; alanine, Ala; valine, Val; and phenylalanine, Phe) and two acidic amino acids (aspartic acid, Asp; and glutamic acid, Glu), were attached to the 4′-position of the tricin B ring. First, treatment of the amino acid *tert*-butyl esters with bis(4-nitrophenyl)carbonate was carried out in the presence of DIPEA for 12 h, and then, tricin and the additional amine were added to the reaction mixture. The reaction was carried out successfully at room temperature, and the desired products **6a**−**f** were obtained after the usual workup of the reaction mixtures. The *tert*-butyl groups of the products were deprotected with trifluoroacetic acid (TFA) to yield the tricin−amino acid conjugates **7a**−**f** (Scheme 2).

In addition, we synthesized the tricin−dipeptide conjugates T-Ala-Asp and T-Ala-Glu from **7b** using the peptide-coupling reagent water-soluble carbodiimide (WSC) and 1-hydroxybenzotriazole (HOBt).[40] Deprotections were carried out using TFA (Scheme 3). Structures were elucidated through IR, ^{1}H and ^{13}C NMR, correlation spectroscopy, heteronuclear multiple quantum coherence, heteronuclear multiple bond correlation, MS, and elemental analysis.

Cell Permeability. We used MDCK cells to investigate transepithelial transport of the synthesized tricin prodrugs (**7a**−**f** and **9a**,**b**) across cell monolayers. MDCK cells have properties resembling Caco-2 cells, which are known to express the peptide

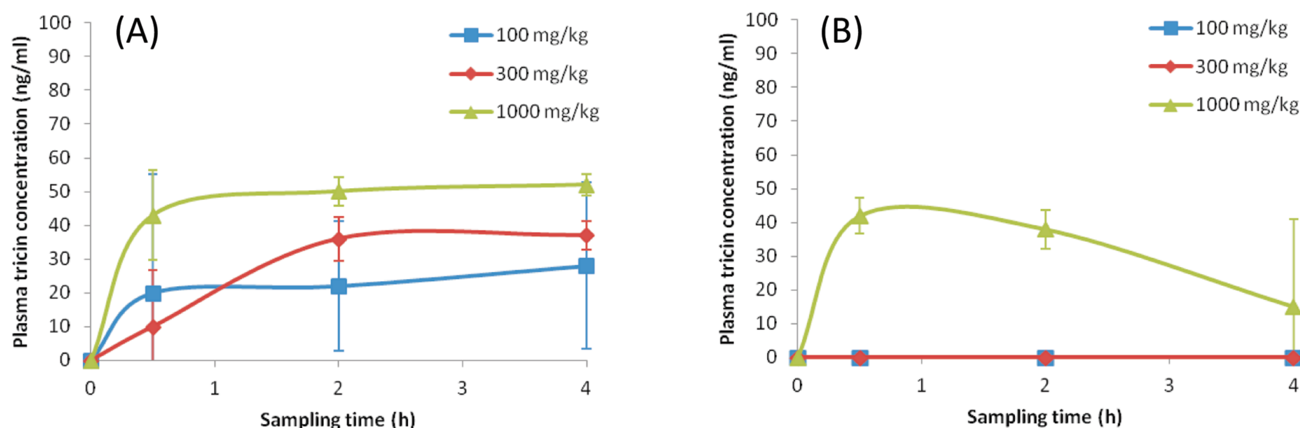
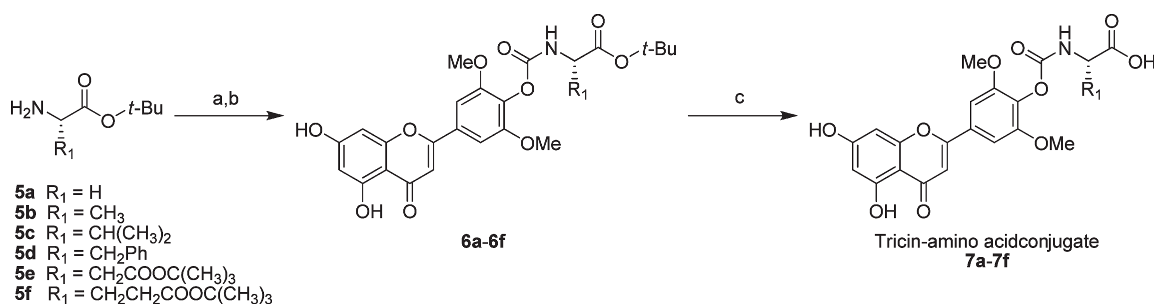


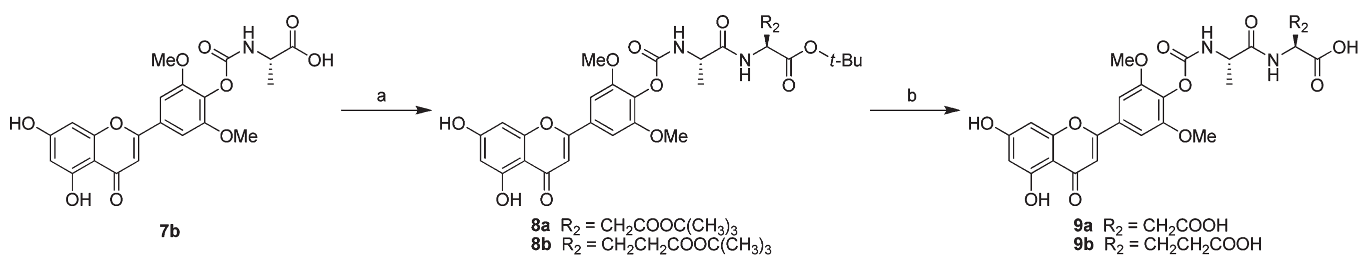
Figure 1. Plasma tricin concentration after oral administration of tricin in Crl:CD (SD) rats. (A) Males and (B) females (means ± SEMs, n = 3).

Scheme 2. Synthesis of Tricin−Amino Acid Conjugates 7a−f[a]



[a] Reagents and conditions: (a) $(4\text{-}NO_2\text{−}PhO)_2CO$, DIPEA, 0 °C → room temperature, 12 h. (b) Tricin **4**, DIPEA, room temperature, 12 h. (c) TFA, DCM, 0 °C → room temperature, 4 h.

Scheme 3. Synthesis of Tricin−Dipeptide Conjugates 9a and 9[a]



[a] Reagents and conditions: (a) Amino acid *tert*-butyl ester, WSC, HOBt, DMF, 0 °C, 3 days. (b) TFA, DCM, 0 °C → room temperature, 4 h.

transporter PepT1 and form tight layers on membrane filters of transport analysis plates.[41,42] The prepared MDCK monolayers were checked by measuring Lucifer yellow rejection after the transport experiments.

Figure 2 indicates that the MDCK cell permeabilities of all of the tested prodrugs [T-Gly (**7a**), T-Ala (**7b**), T-Val (**7c**), T-Phe (**7d**), T-Asp (**7e**), T-Glu (**7f**), T-Ala-Asp (**9a**), and T-Ala-Glu (**9b**)] increased after attaching amino acids and dipeptides to tricin. Among the prodrugs, the tricin−dipeptide conjugate **9b** (T-Ala-Glu) showed an excellent relative permeation rate of 6.3. On the basis of a previous report that the quercetin−dipeptide conjugate (Q-Ala-Glu as a regioisomeric mixture of the 3′-ester and 4′-ester of the quercetin B ring) had good cell permeability,[29] we prepared and evaluated Q-Ala-Glu also. The MDCK cell permeability of Q-Ala-Glu was 4.35 times higher than that of quercetin. These observations strongly suggest that introduction of Ala-Glu dipeptide into tricin may improve tricin permeability into cells and that T-Ala-Glu is a strong prodrug candidate for tricin.

In mammals, the peptide transporter PepT1 belongs to the proton-coupled oligopeptide transporter (POT) family, which is a high-capacity low-affinity transporter. PepT1 has nutritional importance because of its role in intestinal absorption of small peptides from the diet and in reabsorption of peptide-bound amino nitrogen from glomerular filtrate in the kidneys.[43] PepT1 also has significance due to its ability to transport therapeutic agents (e.g., β-lactam antibiotics, antiviral nucleoside prodrugs) and peptidomimetics (e.g., 5-aminolevulinic acid) into cells. Researchers have previously reported that modified Ala-Asp- and Ala-Glu-benzyl esters as model prodrugs have an affinity for

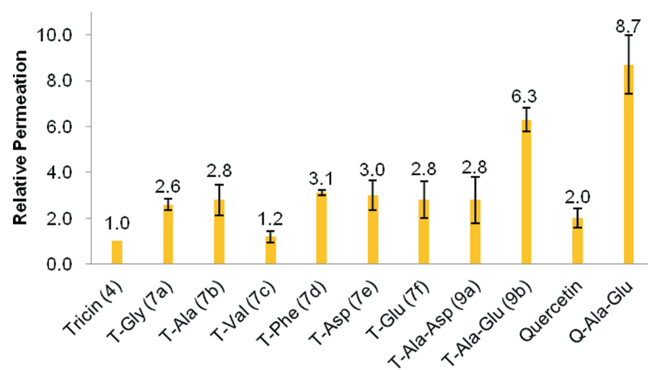


Figure 2. Relative permeabilities of tricin and quercetin prodrugs in MDCK cells (means ± SEMs, $n = 3$).

Table 1. Half-Lives ($t_{1/2}$) of Tricin and Quercetin Prodrugs in MDCK Cell Lysate (Means ± SEMs, $n = 3$)

compound	half-life ($t_{1/2}$) (min)
T-Ala-Glu (**9b**)	165
Q-Ala-Glu	110

PepT1 and efficient permeabilities.[44,45] Thus, this peptide transporter likely improves the oral absorption of T-Ala-Glu.

Stability. Release of the model drugs by hydrolysis of the carbamate linkages in the model prodrugs was examined by using various endogenous hydrolysis enzymes in cells. The time required for 50% release of the model drugs ($t_{1/2}$; half-life) after treatment with cell lysate was determined.

We lysed MDCK cells with lysis buffer and prepared whole MDCK cell lysates. T-Ala-Glu had a half-life of 165 min, which was longer than that of Q-Ala-Glu (Table 1). This was likely caused by the low affinity of T-Ala-Glu for the enzyme active site. T-Ala-Glu has greater steric hindrance due to the existence of bulky functional groups (methoxy groups)[37] around the carbamate linkage as compared to Q-Ala-Glu. This in vitro metabolism experiment indicates that T-Ala-Glu exhibits strong resistance against enzymatic hydrolysis and is more stable than Q-Ala-Glu in the cell lysate. The longer half-life and the higher stability of T-Ala-Glu in the cell lysate may ensure sufficient stability during the transport process in vivo.

Bioavailability of the T-Ala-Glu after Oral Administration in Vivo. On the basis of the results described above, we evaluated tricin in plasma after oral administration of T-Ala-Glu in male rats. Crl:CD (SD) male rats received an oral bolus of 100, 300, or 1000 mg kg^{-1} of T-Ala-Glu in vivo. Blood was collected at 0, 0.5, 2, 4, and 8 h after T-Ala-Glu administration. Tricin derived from parent T-Ala-Glu in plasma was analyzed by RP-HPLC.[32]

In all groups, plasma tricin concentrations after administration of T-Ala-Glu were substantially higher than after administration of intact tricin (Figures 1 and 3 and Table 2). Even in the 100 mg kg^{-1} group, the maximum concentration of tricin was 2497 ng mL^{-1} (7.2 nmol mL^{-1}). In the 300 and 1000 mg kg^{-1} groups, the tricin concentrations exceeded 2800 ng mL^{-1} (8.5 nmol mL^{-1}), about 45 times higher than after administration of intact tricin. These results suggest that T-Ala-Glu was actively absorbed through interaction with PepT1 and that inactivation by various metabolic enzymes was prevented. In addition, T-Ala-Glu demonstrated a bimodal time course in the plasma profile; the in

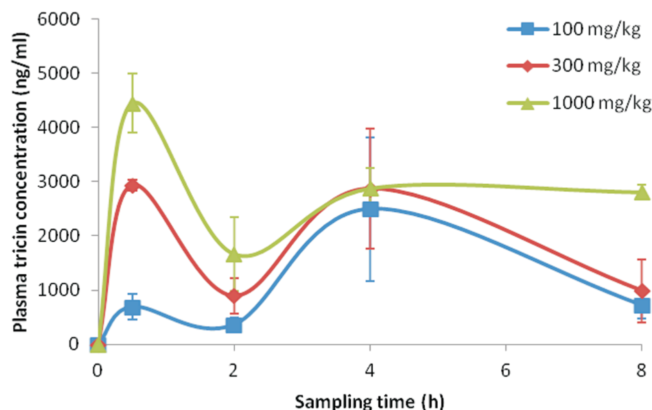


Figure 3. Plasma tricin concentration after oral administration of T-Ala-Glu in Crl:CD (SD) male rats (means ± SEMs, $n = 3$).

Table 2. Pharmacokinetics Parameters of Tricin after Oral Administration of T-Ala-Glu in Crl:CD (SD) Male Rats

dose (mg/kg)	C_{max} 1 (ng/mL)	C_{max} 2 (ng/mL)	AUC^{a}_{0-8} (ng h/mL)
100	692	2497	10261
300	2932	2869	15077
1000	4448	2876	21525

[a] AUC, area under the concentration curve.

vivo absorption experiment revealed the existence of double-site absorption. This phenomenon in Figure 3 implies the possibility of reabsorption by enterohepatic circulation and intestinal absorption, because PepT1 is expressed in the GIT and kidney.[43] However, details of this mechanism are still ambiguous, and further in-depth studies are required.

Recently, Cai et al. reported a study of the administration of 3′,4′,5,5′,7-pentamethoxyflavone (4′,5,7-tri-O-methylated tricin) orally to mice at a single dose, 300 mg kg^{-1} by gavage.[28] The maximum concentration in plasma was 1452 ng mL^{-1} (4.4 nmol mL^{-1}). In contrast, T-Ala-Glu in our study reached approximately twice this concentration.

In addition, we examined the oral toxicity effects of T-Ala-Glu over 14 days with repeated single doses of 300 mg kg^{-1} day^{-1} in Crlj:CD1 (ICR) mice. No abnormal findings were evident in general appearance, body weight, food consumption, hematology, blood chemistry, or autopsy tests (data not shown). Thus, we conclude that T-Ala-Glu is safe for oral administration.

In the present study, we endeavored to improve the oral bioavailability of tricin via a prodrug approach. Our work highlighted the importance of tricin−amino acid derivatives as oral administration forms of tricin. Among them, T-Ala-Glu appears to be a good candidate for development of a new anti-HCMV drug.

CONCLUSIONS

Because tricin was not found to be orally available in its native form, tricin−amino acid derivatives were prepared as prodrugs, and their bioavailabilities were evaluated in vitro and in vivo. Among the prodrugs, the T-Ala-Glu exhibited enhanced permeability, stability in MDCK cells, and excellent bioavailability after oral administration in Crl:CD (SD) male rats, with good safety. Our findings suggest that T-Ala-Glu may be beneficial as a

substantially improved oral administration form and useful in future research toward development of a new anti-HCMV drug.

■ EXPERIMENTAL SECTION

General Chemistry Methods. All solvents and reagents were purchased from the suppliers and used without further purification. IR spectra were recorded on a JASCO FT/IR-460 Plus spectrophotometer (Tokyo, Japan). ^{1}H (600 and 400 MHz) and ^{13}C (150 and 100 MHz) NMR spectra were recorded with a JEOL ECA 600 spectrometer and a JEOL ECX 400 spectrometer (Tokyo, Japan) with tetramethylsilane as an internal standard. MS spectra were obtained using a JEOL JMS-700/GI spectrometer. UV−vis absorption was measured using a PerkinElmer EnVision microplate-reader (Waltham, MA). The used HPLC and microrefrigerated centrifuge were manufactured by Shimadzu Corp. (Kyoto, Japan) and Kubota Co., Ltd. (Tokyo, Japan), respectively. Silica gel column chromatography (CC) was performed on silica gel N-60 (40−50 μm). Thin-layer chromatography (TLC) spots on plates pre-coated with silica gel 60 F_{254} were detected with a UV lamp (254 nm). Fractionations for all CCs were based on TLC analyses. The purity of tricin was checked using RP-HPLC[32] method and confirmed to be 99%. Tricin prodrugs were checked by combustion analysis, and the purity of each compound was ≥95%.

Tricin (4). Reaction of 4-*O*-*tert*-butyldimethylsilyl-3,5-dimethoxybenzoate **1** (152.5 g, 0.47 mol) with LiHMDS (1.0 M solution in THF; 3.76 L, 3.76 mol) and 2′,4′-*O*-bis(*tert*-butyldimethylsilyl)-6′-hydroxyacetophenone **2** (279.7 g, 0.94 mol) in THF (2.0 L) at −78 °C and brought to room temperature for 3 days yielded intermediate **3**. The reaction mixture was partitioned with EtOAc and washed three times with 5% aqueous HCl and once with brine. The organic phase was dried over Na_2SO_4, filtered, and evaporated in vacuo. The residue was dried under vacuum overnight, and the completely dry residue was stirred with 0.5% H_2SO_4 in acetic acid (1.0 L) at 100 °C overnight. The reaction mixture was evaporated, MeOH was added, and the insoluble matter was filtered and washed with MeOH to afford tricin **4** (105.0 g, yield: 68%) as a yellow amorphous powder. ^{1}H NMR (400 MHz; acetone-d_6): δ 7.39 (2H, s, H-2′ and H-6′), 6.74 (1H, s, H-3), 6.56 (1H, d, J = 2.0 Hz, H-8), 6.26 (1H, d, J = 2.0 Hz, H-6), 3.97 (6H, s, 3′- and 5′-OMe). ^{13}C NMR (100 MHz; acetone-d_6): δ 183.1, 165.1, 164.9, 163.3, 158.8, 149.1 (2C), 140.9, 122.3, 105.4 (2C), 105.2, 104.7, 99.7, 94.9, 56.9 (2C). FABMS: m/z = 331 [M + H]$^+$.

Amino Acid *tert*-Butyl Esters of Tricin 6a−f. DIPEA (0.66 mmol) was added to a stirred solution of amino acid *tert*-butyl esters **5a−f** (0.33 mmol) and bis(4-nitrophenyl)carbonate (0.33 mmol) in THF (5 mL) at 0 °C, and stirring was continued for 12 h at room temperature. Tricin **4** (0.30 mmol) and additional DIPEA (0.66 mmol) were added to the reaction mixture at room temperature. After the mixture was stirred for 12 h, the reaction mixture was evaporated, and the residue was purified by silica gel CC eluted with $CHCl_3$/MeOH (50/1) to yield amino acid *tert*-butyl esters of tricin **6a−f**.

*4′-O-CO-(Gly-OtBu)-tricin (**6a**).* Yellow amorphous powder, 63% yield. IR (film): 3435, 1650, 1621, 1603, 1365, 1159, 645 cm^{-1}. ^{1}H NMR (400 MHz; acetone-d_6): δ 7.37 (2H, s, H-2′ and H-6′), 7.00 (1H, br t, J = 6.0 Hz, NH), 6.82 (1H, s, H-3), 6.54 (1H, d, J = 1.8 Hz, H-8), 6.24 (1H, d, J = 1.8 Hz, H-6), 3.97 (6H, s, 3′- and 5′-OMe), 3.84 (2H, d, J = 6.0 Hz, H-2″), 1.46 (9H, s, *t*Bu). ^{13}C NMR (100 MHz; acetone-d_6): δ 182.3, 168.8, 164.2, 163.4, 162.5, 158.0, 153.9, 153.7 (2C), 132.4, 129.0, 105.5, 104.7, 103.6 (2C), 99.0, 94.2, 80.9, 56.1 (2C), 43.5, 28.8 (3C). FABMS: m/z = 488 [M + H]$^+$. Anal. calcd for $C_{24}H_{25}NO_{10}$: C, 59.13; H, 5.17; N, 2.87. Found: C, 59.15; H, 5.18; N, 2.88.

*4′-O-CO-(Ala-OtBu)-tricin (**6b**).* Yellow amorphous powder, 55% yield. IR (film): 3433, 1650, 1619, 1602, 1363, 1162, 644 cm^{-1}. ^{1}H NMR (400 MHz; acetone-d_6): δ 7.36 (2H, s, H-2′ and H-6′), 7.00 (1H, br d, J = 9.2 Hz, NH), 6.81 (1H, s, H-3), 6.53 (1H, d, J = 2.3 Hz, H-8), 6.23 (1H, d, J = 2.3 Hz, H-6), 4.14 (1H, quint, J = 7.3 Hz, H-2″), 3.92 (6H, s, 3′- and 5′-OMe), 1.46 (9H, s, *t*Bu), 1.41 (3H, d, J = 7.3 Hz, H-3″). ^{13}C NMR (100 MHz; acetone-d_6): δ 183.2, 172.5, 165.0, 164.3, 163.3, 158.8, 154.5 (2C), 153.9, 133.3, 129.8, 106.4, 105.5, 104.5 (2C), 99.8, 95.0, 81.6, 56.9 (2C), 51.6, 28.1 (3C), 18.0. FABMS: m/z = 502 [M + H]$^+$. Anal. calcd for $C_{25}H_{27}NO_{10}$: C, 59.88; H, 5.43; N, 2.79. Found: C, 59.92; H, 5.41; N, 2.83.

*4′-O-CO-(Val-OtBu)-tricin (**6c**).* Yellow amorphous powder, 52% yield. IR (film): 3343, 1654, 1625, 1595, 13637, 1163, 694 cm^{-1}. ^{1}H NMR (400 MHz; acetone-d_6): δ 7.36 (2H, s, H-2′ and H-6′), 6.87−6.83 (1H, m, NH), 6.80 (1H, s, H-3), 6.53 (1H, d, J = 2.0 Hz, H-8), 6.23 (1H, d, J = 2.0 Hz, H-6), 4.10−4.04 (1H, m, H-2″), 3.92 (6H, s, 3′- and 5′-OMe), 2.26−2.14 (1H, m, H-3″), 1.48 (9H, s, *t*Bu), 1.04−1.00 (6H, m, 2Me of Val). ^{13}C NMR (100 MHz; acetone-d_6): δ 182.3, 170.6, 164.2, 163.4, 162.5, 158.0, 153.6 (3C), 132.5, 128.9, 105.5, 104.7, 103.7 (2C), 99.0, 94.2, 81.0, 60.5, 56.0 (2C), 31.1, 27.4 (3C), 18.6, 17.3. FABMS: m/z = 530 [M + H]$^+$. Anal. calcd for $C_{27}H_{31}NO_{10}$: C, 61.24; H, 5.90; N, 2.65. Found: C, 61.31; H, 5.95; N, 2.56.

*4′-O-CO-(Phe-OtBu)-tricin (**6d**).* Yellow amorphous powder, 51% yield. IR (film): 3388, 1654, 1617, 1594, 1364, 1162, 697 cm^{-1}. ^{1}H NMR (400 MHz; acetone-d_6): δ 7.36 (2H, s, H-2′ and H-6′), 7.36−7.30 (4H, m, Ar of Phe), 7.28−7.23 (1H, m, Ar of Phe), 6.92−6.88 (1H, m, NH), 6.81 (1H, s, H-3), 6.55 (1H, d, J = 1.9 Hz, H-8), 6.24 (1H, d, J = 1.9 Hz, H-6), 4.40−4.34 (1H, m, H-2″), 3.90 (6H, s, 3′- and 5′-OMe), 3.19 (1H, dd, J = 13.7, 6.0 Hz, H-3″), 3.08 (1H, dd, J = 13.7, 6.0 Hz, H-3″), 1.42 (9H, s, *t*Bu). ^{13}C NMR (100 MHz; acetone-d_6): δ 183.2, 171.2, 165.8, 164.4, 163.4, 158.9, 154.5 (2C), 153.8, 138.1, 133.2, 130.4 (2C), 129.2 (2C), 127.5, 126.9, 106.4, 104.5 (3C), 99.9, 95.0, 82.0, 57.2, 56.9 (2C), 38.3, 28.1 (3C). FABMS: m/z = 578 [M + H]$^+$. Anal. calcd for $C_{31}H_{31}NO_{10}$: C, 64.46; H, 5.41; N, 2.43. Found: C, 64.34; H, 5.30; N, 2.55.

*4′-O-CO-[Asp(OtBu)-OtBu]-tricin (**6e**).* Yellow amorphous powder, 53% yield. IR (film): 3343, 1654, 1616, 1593, 1365, 1161, 694 cm^{-1}. ^{1}H NMR (400 MHz; acetone-d_6): δ 7.37 (2H, s, H-2′ and H-6′), 6.98−6.93 (1H, m, NH), 6.81 (1H, s, H-3), 6.54 (1H, d, J = 2.0 Hz, H-8), 6.24 (1H, d, J = 2.0 Hz, H-6), 4.49−4.44 (1H, m, H-2″), 3.93 (6H, s, 3′- and 5′-OMe), 2.83 (1H, dd, J = 16.0, 6.0 Hz, H-3″), 2.75 (1H, dd, J = 16.0, 6.0 Hz, H-3″), 1.47 (9H, s, *t*Bu), 1.46 (9H, s, *t*Bu). ^{13}C NMR (100 MHz; acetone-d_6): δ 182.3, 169.6, 169.5, 164.2, 163.5, 163.4, 162.5, 158.0, 153.7 (2C), 132.4, 129.1, 105.6, 104.7, 103.7 (2C), 99.0, 94.2, 81.4, 80.7, 56.1 (2C), 51.9, 37.5, 27.4 (3C), 27.3 (3C). FABMS: m/z = 602 [M + H]$^+$. Anal. calcd for $C_{30}H_{35}NO_{12}$: C, 59.89; H, 5.86; N, 2.33. Found: C, 60.03; H, 5.95; N, 2.45.

*4′-O-CO-[Glu(OtBu)-OtBu]-tricin (**6f**).* Yellow amorphous powder, 61% yield. IR (film): 3419, 1649, 1619, 1605, 1366, 1159, 695 cm^{-1}. ^{1}H NMR (400 MHz; acetone-d_6): δ 7.36 (2H, s, H-2′ and H-6′), 7.01−6.97 (1H, m, NH), 6.80 (1H, s, H-3), 6.53 (1H, d, J = 2.0 Hz, H-8), 6.23 (1H, d, J = 2.0 Hz, H-6), 4.24−4.18 (1H, m, H-2″), 3.93 (6H, s, 3′- and 5′-OMe), 2.45−2.41 (2H, m, H-4″), 2.20−2.11 (1H, m, H-3″), 1.98−1.89 (1H, m, H-3″), 1.47 (9H, s, *t*Bu), 1.44 (9H, s, *t*Bu). ^{13}C NMR (100 MHz; acetone-d_6): δ 182.3, 171.6, 170.8, 164.2, 163.5, 163.4, 162.5, 158.0, 153.6 (2C), 153.4, 132.5, 129.0, 105.5, 104.7, 103.7 (2C), 99.0, 94.2, 81.1, 79.8, 56.1 (2C), 54.4, 31.1, 27.5 (3C), 27.3 (3C). FABMS: m/z = 616 [M + H]$^+$. Anal. calcd for $C_{31}H_{37}NO_{12}$: C, 60.48; H, 6.06; N, 2.28. Found: C, 60.61; H, 6.01; N, 2.35.

Amino Acid Conjugates of Tricin 7a−f. TFA (2 mL) was added to a stirred solution of amino acid *tert*-butyl esters of tricin **6a−f** (0.082 mmol) in dichloromethane (DCM; 5 mL) at 0 °C, and stirring was continued for 4 h at room temperature. The reaction mixture was evaporated, and the residue was suspended in DCM (5 mL) and filtered, and the filtrate was washed with DCM (5 mL) to yield the amino acid conjugates of tricin **7a−f**.

*4′-O-CO-Gly-tricin (**7a**).* Yellow amorphous powder, 94% yield. IR (film): 3294, 1715, 1656, 1639, 1612, 1354, 1166, 663 cm^{-1}. ^{1}H NMR

(400 MHz; DMSO-d_6): δ 10.86 (1H, br s, COOH), 8.05 (1H, t, J = 6.0 Hz, NH), 7.34 (2H, s, H-2′ and H-6′), 7.09 (1H, s, H-3), 6.56 (1H, d, J = 1.9 Hz, H-8), 6.20 (1H, d, J = 1.9 Hz, H-6), 3.85 (6H, s, 3′- and 5′-OMe), 3.72 (2H, d, J = 6.0 Hz, H-2″). ^{13}C NMR (100 MHz; DMSO-d_6): δ 182.5, 171.7, 164.9, 163.3, 161.9, 158.0, 154.1, 153.5 (2C), 132.1, 128.9, 106.1, 104.5, 104.2 (2C), 99.5, 94.9, 56.9 (2C), 42.9. FABMS: m/z = 432 $[M + H]^+$. Anal. calcd for $C_{20}H_{17}NO_{10}$: C, 55.69; H, 3.97; N, 3.25. Found: C, 55.39; H, 3.94; N, 3.32.

*4′-O-CO-Ala-tricin (**7b**).* Yellow amorphous powder, 81% yield. IR (film): 3306, 1714, 1649, 1616, 1599, 1361, 1132, 639 cm^{-1}. ^{1}H NMR (400 MHz; DMSO-d_6): δ 10.86 (1H, br s, COOH), 8.11 (1H, d, J = 7.8 Hz, NH), 7.33 (2H, s, H-2′ and H-6′), 7.08 (1H, s, H-3), 6.56 (1H, d, J = 1.9 Hz, H-8), 6.20 (1H, d, J = 1.9 Hz, H-6), 4.03 (1H, quint, J = 7.3 Hz, H-2″), 3.84 (6H, s, 3′- and 5′-OMe), 1.29 (3H, d, J = 7.3 Hz, H-3″). ^{13}C NMR (100 MHz; DMSO-d_6): δ 182.5, 174.5, 164.9, 163.3, 161.9, 158.0, 153.5 (3C), 132.2, 128.8, 106.1, 104.5, 104.2 (2C), 99.5, 94.9, 57.0 (2C), 50.0, 17.8. FABMS: m/z = 446 $[M + H]^+$. Anal. calcd for $C_{21}H_{19}NO_{10}$: C, 56.63; H, 4.30; N, 3.14. Found: C, 56.94; H, 4.24; N, 3.12.

*4′-O-CO-Val-tricin (**7c**).* Yellow amorphous powder, 90% yield. IR (film): 3320, 1714, 1656, 1619, 1600, 1364, 1162, 642 cm^{-1}. ^{1}H NMR (400 MHz; DMSO-d_6): δ 10.91 (1H, br s, COOH), 8.06 (1H, d, J = 9.2 Hz, NH), 7.38 (2H, s, H-2′ and H-6′), 7.12 (1H, s, H-3), 6.59 (1H, d, J = 1.9 Hz, H-8), 6.23 (1H, d, J = 1.9 Hz, H-6), 3.95−3.88 (1H, m, H-2″), 3.88 (6H, s, 3′- and 5′-OMe), 2.15−2.08 (1H, m, H-3″), 0.96 (3H, d, J = 3.8 Hz, 3″-Me), 0.94 (3H, d, J = 3.8 Hz, 3″-Me). ^{13}C NMR (100 MHz; DMSO-d_6): δ 182.0, 172.9, 164.4, 162.9, 161.4, 157.5, 153.7, 153.0 (2C), 131.8, 128.3, 105.6, 104.0, 103.7 (2C), 99.0, 94.4, 59.8, 56.4 (2C), 30.0, 19.1, 18.0. FABMS: m/z = 474 $[M + H]^+$. Anal. calcd for $C_{23}H_{23}NO_{10}$: C, 58.35; H, 4.90; N, 2.96. Found: C, 58.23; H, 4.82; N, 2.87.

*4′-O-CO-Phe-tricin (**7d**).* Yellow amorphous powder, 80% yield. IR (film): 3310, 1713, 1655, 1612, 1602, 1358, 1164, 646 cm^{-1}. ^{1}H NMR (400 MHz; DMSO-d_6): δ 10.86 (1H, br s, COOH), 8.15 (1H, d, J = 8.2 Hz, NH), 7.30 (2H, s, H-2′ and H-6′), 7.29−7.20 (5H, m, Ar of Phe), 7.07 (1H, s, H-3), 6.55 (1H, d, J = 1.9 Hz, H-8), 6.19 (1H, d, J = 1.9 Hz, H-6), 4.20−4.13 (1H, m, H-2″), 3.78 (6H, s, 3′- and 5′-OMe), 3.08 (1H, dd, J = 13.7, 4.6 Hz, H-3″), 2.87 (1H, dd, J = 13.7, 4.6 Hz, H-3″). ^{13}C NMR (100 MHz; DMSO-d_6): δ 182.5, 173.5, 164.9, 163.3, 161.9, 158.0, 153.7, 153.4 (2C), 138.2, 132.2, 129.7 (2C), 128.8 (3C), 127.0, 106.1, 104.5, 104.2 (2C), 99.5, 94.9, 56.9 (2C), 56.2, 37.0. FABMS: m/z = 522 $[M + H]^+$, 504 $[M + H - H_2O]^+$. Anal. calcd for $C_{27}H_{19}NO_{10}$: C, 62.19; H, 4.45; N, 2.69. Found: C, 62.33; H, 4.31; N, 2.60.

*4′-O-CO-Asp-tricin (**7e**).* Yellow amorphous powder, 90% yield. IR (film): 3309, 1721, 1654, 1610, 1601, 1359, 1165, 645 cm^{-1}. ^{1}H NMR (400 MHz; DMSO-d_6): δ 10.90 (2H, br s, COOH), 8.15 (1H, d, J = 8.2 Hz, NH), 7.37 (2H, s, H-2′ and H-6′), 7.12 (1H, s, H-3), 6.59 (1H, d, J = 1.9 Hz, H-8), 6.23 (1H, d, J = 1.9 Hz, H-6), 4.35 (1H, q, J = 7.8 Hz, H-2″), 3.88 (6H, s, 3′- and 5′-OMe), 2.77 (1H, dd, J = 16.5, 6.7 Hz, H-3″), 2.63 (1H, dd, J = 16.5, 6.7 Hz, H-3″). ^{13}C NMR (100 MHz; DMSO-d_6): δ 182.0, 172.3, 171.6, 164.4, 162.8, 161.4, 157.5, 153.0, 152.9 (2C), 131.7, 128.3, 105.6, 104.0, 103.8 (2C), 99.0, 94.4, 56.4 (2C), 55.8, 29.6. FABMS: m/z = 490 $[M + H]^+$. Anal. calcd for $C_{22}H_{19}NO_{12}$: C, 53.99; H, 3.91; N, 2.86. Found: C, 53.69; H, 3.89; N, 2.86.

*4′-O-CO-Glu-tricin (**7f**).* Yellow amorphous powder, 83% yield. IR (film): 3299, 1719, 1650, 1614, 1601, 1358, 1165, 645 cm^{-1}. ^{1}H NMR (400 MHz; DMSO-d_6): δ 10.93 (2H, br s, COOH), 8.15 (1H, d, J = 8.2 Hz, NH), 7.38 (2H, s, H-2′ and H-6′), 7.12 (1H, s, H-3), 6.60 (1H, d, J = 2.0 Hz, H-8), 6.24 (1H, d, J = 2.0 Hz, H-6), 4.09−4.02 (1H, m, H-2″), 3.89 (6H, s, 3′- and 5′-OMe), 2.40−2.35 (2H, m, H-4″), 2.09−2.00 (1H, m, H-3″), 1.88−1.78 (1H, m, H-3″). ^{13}C NMR (100 MHz; DMSO-d_6): δ 182.5, 174.3, 173.6, 164.9, 163.3, 161.9, 158.0, 153.9, 153.4 (2C), 132.3, 128.8, 106.1, 104.5, 104.2 (2C), 94.9 (2C), 56.9 (2C), 53.7, 30.4, 26.9. FABMS: m/z = 504 $[M + H]^+$. Anal. calcd for $C_{23}H_{21}NO_{12}$: C, 54.87; H, 4.20; N, 2.78. Found: C, 54.62; H, 4.28; N, 2.84.

Dipeptide *tert*-Butyl Esters of Tricin 8a and 8b. 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (0.34 mmol) and 1-hydroxy-1*H*-benzotriazole (0.62 mmol) were added to a stirred solution of the alanine conjugate of tricin **7b** (0.31 mmol) and amino acid *tert*-butyl esters **5e** and **5f** (0.31 mmol) in *N,N*-dimethylformamide (DMF; 5 mL) at 0 °C, and stirring was continued for 3 days. The reaction mixture was evaporated, and the residue was purified by silica gel column CC eluted with $CHCl_3$ to yield dipeptide *tert*-butyl esters of tricin **8a** and **8b**.

*4′-O-CO-Ala-[Asp(OtBu)-OtBu]-tricin (**8a**).* Yellow amorphous powder, 32% yield. IR (film): 3406, 1656, 1619, 1603, 1366, 1160, 633 cm^{-1}. ^{1}H NMR (400 MHz; acetone-d_6): δ 7.51 (1H, br d, J = 8.2 Hz, NH), 7.34 (2H, s, H-2′ and H-6′), 7.00 (1H, br d, J = 7.3 Hz, NH), 6.80 (1H, s, H-3), 6.53 (1H, d, J = 1.9 Hz, H-8), 6.23 (1H, d, J = 1.9 Hz, H-6), 4.68−4.63 (1H, m, H-2‴), 4.30 (1H, quint, J = 6.8 Hz, H-2″), 3.91 (6H, s, 3′- and 5′-OMe), 2.74 (2H, d, J = 5.5 Hz, H-3‴), 1.44 (9H, s, *t*Bu), 1.43 (3H, d, J = 6.8 Hz, H-3″), 1.40 (9H, s, *t*Bu). ^{13}C NMR (100 MHz; acetone-d_6): δ 182.3, 171.9, 169.6 (3C), 164.3, 163.4, 162.5, 158.0, 153.6 (2C), 131.9, 129.0, 105.5, 104.7, 103.5 (2C), 99.0, 94.3, 81.3, 80.6, 56.0 (2C), 50.8, 49.5, 37.3, 27.4 (3C), 27.3 (3C), 18.1. FABMS: m/z = 673 $[M + H]^+$. Anal. calcd for $C_{33}H_{40}N_2O_{13}$: C, 58.92; H, 5.99; N, 4.16. Found: C, 58.91; H, 5.92; N, 4.12.

*4′-O-CO-Ala-[Glu(OtBu)-OtBu]-tricin (**8b**).* Yellow amorphous powder, 33% yield. IR (film): 3343, 1657, 1627, 1601, 1366, 1160, 633 cm^{-1}. ^{1}H NMR (400 MHz; acetone-d_6): δ 7.46 (1H, br d, J = 7.8 Hz, NH), 7.34 (2H, s, H-2′ and H-6′), 6.97 (1H, br d, J = 7.8 Hz, NH), 6.80 (1H, s, H-3), 6.53 (1H, d, J = 1.9 Hz, H-8), 6.24 (1H, d, J = 1.9 Hz, H-6), 4.42−4.36 (1H, m, H-2‴), 4.29 (1H, quint, J = 7.3 Hz, H-2″), 3.90 (6H, s, 3′- and 5′-OMe), 2.34−2.30 (2H, m, H-4‴), 2.14−2.05 (1H, m, H-3‴), 1.92−1.82 (1H, m, H-3‴), 1.45 (9H, s, *t*Bu), 1.43 (3H, d, J = 7.3 Hz, H-3″), 1.38 (9H, s, *t*Bu). ^{13}C NMR (100 MHz; acetone-d_6): δ 182.3, 172.1, 171.5, 170.7, 164.3, 163.4, 162.5, 158.0, 153.6 (2C), 153.1, 132.0, 128.9, 105.4, 104.7, 103.5 (2C), 99.0, 94.3, 81.2, 79.7, 56.0 (2C), 52.3, 50.7, 31.2, 27.4 (3C), 27.3 (3C), 27.2, 18.0. FABMS: m/z = 687 $[M + H]^+$. Anal. calcd for $C_{34}H_{42}N_2O_{13}$: C, 59.47; H, 6.16; N, 4.08. Found: C, 59.61; H, 6.11; N, 4.10.

Dipeptide Conjugates of Tricin 9a and 9b. TFA (2 mL) was added to a stirred solution of the dipeptide *tert*-butyl esters of tricin **8a** and **8b** (0.095 mmol) in DCM (5 mL) at 0 °C, and stirring was continued for 4 h at room temperature. The reaction mixture was evaporated, and the residue was suspended in DCM (5 mL) and filtered, and the filtrate was washed with DCM (5 mL) to yield the dipeptide conjugates of tricin **9a** and **9b**.

*4′-O-CO-Ala-Asp-tricin (**9a**).* Yellow amorphous powder, 91% yield. IR (film): 3372, 1722, 1655, 1611, 1601, 1359, 1164, 663 cm^{-1}. ^{1}H NMR (600 MHz; DMSO-d_6): δ 10.91 (2H, br s, COOH), 8.15 (1H, d, J = 8.2 Hz, NH), 7.99 (1H, d, J = 7.8 Hz, NH), 7.37 (2H, s, H-2′ and H-6′), 7.12 (1H, s, H-3), 6.59 (1H, d, J = 2.0 Hz, H-8), 6.23 (1H, d, J = 2.0 Hz, H-6), 4.56 (1H, quint, J = 7.8 Hz, H-2‴), 4.13 (1H, quint, J = 6.8 Hz, H-2″), 3.88 (6H, s, 3′- and 5′-OMe), 2.69 (1H, dd, J = 16.5, 6.0 Hz, H-3‴), 2.62 (1H, dd, J = 16.5, 6.0 Hz, H-3‴), 1.29 (3H, d, J = 6.8 Hz, H-3″). ^{13}C NMR (150 MHz; DMSO-d_6): δ 182.0, 172.3, 172.0, 171.6, 164.4, 162.8, 161.4, 157.5, 153.0 (2C), 152.7, 131.7, 128.3, 105.6, 104.0, 103.7 (2C), 99.0, 94.4, 56.4 (2C), 50.1, 48.5, 36.0, 18.4. FABMS: m/z = 561 $[M + H]^+$. Anal. calcd for $C_{25}H_{24}N_2O_{13}$: C, 53.57; H, 4.32; N, 5.00. Found: C, 53.44; H, 4.38; N, 4.95.

*4′-O-CO-Ala-Glu-tricin (**9b**).* Yellow amorphous powder, 96% yield. IR (film): 3296, 1711, 1650, 1619, 1602, 1362, 1161, 645 cm^{-1}. ^{1}H NMR (600 MHz; DMSO-d_6): δ 10.90 (2H, br s, COOH), 8.09 (1H, d, J = 7.8 Hz, NH), 7.98 (1H, d, J = 7.8 Hz, NH), 7.37 (2H, s, H-2′ and H-6′), 7.12 (1H, s, H-3), 6.59 (1H, d, J = 2.0 Hz, H-8), 6.24 (1H, d, J = 2.0 Hz, H-6), 4.28−4.22 (1H, m, H-2‴), 4.12 (1H, quint, J = 7.3 Hz, H-2″), 3.89 (6H, s, 3′- and 5′-OMe), 2.31−2.23 (2H, m, H-4‴), 2.03−1.94 (1H, m, H-3‴), 1.83−1.74 (1H, m, H-3‴), 1.27 (1H, d, J = 7.3 Hz, H-3″). ^{13}C NMR (150 MHz; DMSO-d_6): δ 182.0, 173.7, 173.1,

dx.doi.org/10.1021/jm1015457 | *J. Med. Chem.* 2011, 54, 1529–1536

172.2, 164.4, 162.8, 161.4, 157.5, 153.0 (2C), 152.7, 131.7, 128.3, 105.5, 104.0, 103.7 (2C), 99.0, 94.4, 56.4 (3C), 51.1, 29.9, 26.4, 18.2. FABMS: $m/z = 575$ $[M + H]^+$. Anal. calcd for $C_{26}H_{26}N_2O_{13}$: C, 54.36; H, 4.56; N, 4.88. Found: C, 54.40; H, 4.48; N, 4.92.

Biological Evaluation Methods. *Cell Permeability Evaluations.* MDCK cells were seeded in culture flasks and passaged in minimum essential medium (MEM) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (100 U mL^{-1}). Cells were seeded onto tissue culture-treated transwells (1.12 cm^2, 0.4 μm pore size) at a density of 2.7×10^5 cm^{-2} and grown at 37 °C in a humidified incubator under an atmosphere of 5% CO_2. Transport experiments were performed 2 days after seeding. To evaluate the cell permeability of the prodrugs, the rate of drug transport from apical to basolateral solution was determined. The concentrations of the tested prodrugs were 10, 20, 60, and 100 μM. The prodrugs were diluted with phosphate-buffered saline (PBS) buffer, and 300 μL was added to the filter well of an apical plate. The basolateral medium was 500 μL of MEM. The 12-well plates were incubated under 5% CO_2 atmosphere at 37 °C. After 1 h, 150 μL samples were collected from the apical and basolateral solutions and measured using a UV−vis spectrometer at 330 nm.

Stability Evaluations. MDCK cell lysates were prepared using 1.33×10^6 cells in accordance with the manual.[46] Stability evaluation procedures were carried out as described previously.[32]

Animal Experiments. The experimental and study design were approved by the Committee of Safety Research Institute for Chemical Compounds Co., Ltd. under the Institutional Animal Care Guidelines. All handling and procedures were carried out in accordance with the appropriate Institutional Animal Care Guidelines.

Three week old male and female Crl:CD (SD) rats were purchased from Charles River Laboratories, Inc. (Wilmington, MA). All animals were housed in metal cages (2−3 rats/cage) and had free access to tap water and a basal diet, Charles River Formula-1 (Oriental Yeast Co., Ltd., Tokyo, Japan). The animals were kept in an experimental animal room under controlled conditions of humidity (50 ± 20%), light (12/12 h light/dark cycle), and temperature (22 ± 3 °C). After 2 weeks of quarantine, the animals were divided into three experimental groups. All handing and procedures were carried out in accordance with appropriate Institutional Animal Care Guidelines. Tricin **4** was mixed with 1% aqueous methylcellulose solution (Shin-Etsu Chemical Co., Ltd., Tokyo, Japan) and sonicated to produce a visually homogeneous solution (10 mL kg^{-1}) for gavage. The animals received a single oral dose (100, 300, or 1,000 mg kg^{-1}) by gavage. Blood was collected at 0, 0.5, 2, 4, and 8 h after administration by syringe from the tail of the rat. Plasma was obtained by centrifugation (6000 rpm, 10 min, 4 °C). Biomatrices were held at −25 °C until analysis, no longer than 19 days. Plasma tricin concentrations were analyzed as described previously.[32]

AUTHOR INFORMATION

Corresponding Author

*Tel: +81-58-293-2619. Fax: +81-58-293-2794. E-mail: koketsu@gifu-u.ac.jp (M.K.). Tel: +81-58-230-6555. Fax: +81-58-230-6551. E-mail: kuni@gifu-u.ac.jp (K.W.).

ABBREVIATIONS USED

AMVN, 2,2′-azobis(2,4-dimethylvaleronitrile); DCM, dichloromethane; DIPEA, *N*,*N*-diisopropylethylamine; DMF, *N*,*N*-dimethylformamide; GCV, ganciclovir; GIT, gastrointestinal tract; HCMV, human cytomegalovirus; HOBt, 1-hydroxybenzotriazole; LiHMDS, lithium bis(trimethylsilyl)amide; MDCK, Madin−Darby canine kidney; MEM, minimum essential medium; MRP2, multidrug resistance protein 2; RP-HPLC, reverse-phase high-performance liquid chromatography; TFA, trifluoroacetic acid; THF, tetrahydrofuran; PBS, phosphate-buffered saline; PepT, peptide transporter; POT, proton-coupled oligopeptide transporter; WSC, water-soluble carbodiimide

REFERENCES

(1) Harborne, J. B.; Williamson., C. A. Advances in flavonoid research since 1992. *Phytochemistry* **2000**, *55*, 481–504.

(2) Havsteen, B. H. The biochemistry and medicinal significance of the flavonoids. *Pharmacol. Ther.* **2002**, *96*, 67–202.

(3) Inoue, Y.; Yamaguchi, K.; Take, Y.; Nakamura, S. Inhibition of avian myeloblastosis virus reverse transcriptase by flavones and isoflavones. *J. Antibiot.* **1989**, *42*, 1523–1525.

(4) Ono, K.; Nakane, H.; Fukushima, M.; Chermann, J. C.; Barré-Sinoussi, F. Inhibition of reverse transcriptase activity by a flavonoid compound, 5,6,7-trihydroxyflavone. *Biochem. Biophys. Res. Commun.* **1989**, *160*, 982–987.

(5) Hagiwara, M.; Inoue, S.; Tanaka, T.; Nunoki, K.; Ito, M.; Hidaka, H. Differential effects of flavonoids as inhibitors of tyrosine protein kinases and serine/threonine protein kinases. *Biochem. Pharmacol.* **1988**, *37*, 2987–2992.

(6) Geahlen, R. L.; Koonchanok, N. M.; McLaughlin, J. L.; Pratt, D. E. Inhibition of protein-tyrosine kinase activity by flavonoids and related compounds. *J. Nat. Prod.* **1989**, *52*, 982–986.

(7) Hirano, T.; Oka, K.; Akiba, M. Antiproliferative effects of synthetic and naturally occurring flavonoids on tumor cells of the human breast carcinoma cell line, ZR-75−1. *Res. Commun. Chem. Pathol. Pharmacol.* **1989**, *64*, 69–78.

(8) Le Marchand, L. Cancer preventive effects of flavonoids—A review. *Biomed. Pharmacother.* **2002**, *56*, 296–301.

(9) Cassady, J. M.; Baird, W. H.; Chang, C.-J. Natural products as a source of potential cancer chemotherapeutic and chemopreventive agents. *J. Nat. Prod.* **1990**, *53*, 23–41.

(10) Hatano, T.; Yasuhara, T.; Miyamoto, K.; Okuda, T. Anti-human immunodeficiency virus phenolics from licorice. *Chem. Pharm. Bull.* **1988**, *36*, 2286–2288.

(11) Zhou, J.-M.; Ibrahim, R. K. Tricin—A potential multifunctional nutraceutical. *Phytochem. Rev.* **2010**, *9*, 413–424.

(12) Saleh, N. A. M.; Nozzolillo, C.; Altosaar, I. Flavonoid variations in *Avena* species. *Biochem. Syst. Ecol.* **1988**, *16*, 597–599.

(13) Kong, C. H.; Zhao, H.; Xu, X. H.; Wang, P.; Gu, Y. Activity and allelopathy of soil of flavone *O*-glycosides from rice. *J. Agric. Food Chem.* **2007**, *55*, 6007–6012.

(14) Kwon, Y. S.; Kim, E. Y.; Kim, W. J.; Kim, W. K.; Kim, C. M. Antioxidant constituents from *Setaria viridis*. *Arch. Pharm. Res.* **2002**, *25*, 300–305.

(15) Watanabe, M. Antioxidative phenolic compounds from Japanese barnyard millet (*Echinochloa utilis*) grains. *J. Agric. Food Chem.* **1999**, *47*, 4500–4505.

(16) Yazawa, K.; Tsuchida, Y.; Yamada, R.; Sadanari, H.; Murayama, T. Anti-influenza virus activity by tricin, isolated *Sasa albo-marginata* in Japan. *Antiviral Res.* **2010**, *86*, A49.

(17) Moscatelli, V.; Hnatyszyn, O.; Acevedo, C.; Megias, J.; Alcaraz, M. J.; Ferrana, G. Flavonoids from *Artemisia copa* with anti-inflammatory activity. *Planta Med.* **2006**, *72*, 72–74.

(18) Kuwabara, H.; Mouri, K.; Otsuka, H.; Kasai, R.; Yamasaki, K. Tricin from a Malagasy connaraceous plant with potent antihistamic activity. *J. Nat. Prod.* **2003**, *66*, 1273–1275.

(19) Jeong, Y. H.; Chung, S. Y.; Han, A.-R.; Sung, M. K.; Jang, D. S.; Lee, J.; Kwon, Y.; Lee, H. J.; Seo, E.-K. P-Glycoprotein inhibitory activity of 2 phenolic compounds, (−)-syringaresinol and tricin from *Sasa borealis*. *Chem. Biodiversity* **2007**, *4*, 12–16.

(20) Verschoyle, R. D.; Greaves, P.; Cai, H.; Borkhardt, A.; Broggini, M.; D'Incalci, M.; Riccio, E.; Doppalapudi, R.; Kapetanovic, I. M.; Steward, W. P.; Gescher, A. J. Preliminary safety evaluation of the putative cancer chemopreventive agent tricin, a naturally occurring flavone. *Cancer Chemother. Pharmacol.* **2006**, *57*, 1–6.

(21) Oyama, T.; Yasui, Y.; Sugie, S.; Koketsu, M.; Watanabe, K.; Tanaka, T. Dietary tricin suppresses inflammation-related colon carcinogenesis in male Crj: CD-1 mice. *Cancer Prev. Res.* **2009**, *2*, 1031–1038.

(22) Sakai, A.; Watanabe, K.; Koketsu, M.; Akuzawa, K.; Yamada, R.; Li, Z.; Sadanari, H.; Matsubara, K.; Murayama, T. Anti-human cytomegalovirus activity of constituents from *Sasa albo-marginata* (Kumazasa in Japan). *Antivir. Chem. Chemother.* **2008**, *19*, 125–132.

(23) Zhen, H.; Fang, F.; Ye, D. Y.; Shu, S. N.; Zhou, Y. F.; Dong, Y. S.; Nie, X. C.; Li, G. Experimental study on the action of allitridin against human cytomegalovirus *in vitro*: Inhibitory effects on immediate-early genes. *Antiviral Res.* **2006**, *72*, 68–74.

(24) Formica, J. V.; Regelson, W. Review of the biology of quercetin and related flavonoids. *Food Chem. Toxicol.* **1995**, *33*, 1061–1080.

(25) Olthof, M. R.; Hollman, P. C. H.; Vree, T. B.; Katan, M. B. Bioavailabilities of quercetin-3-glucoside and quercetin-4′-glucoside do not differ in humans. *J. Nutr.* **2000**, *130*, 1200–1203.

(26) Montenegro, L.; Carbone, C.; Maniscalco, C.; Lambusta, D.; Nicolosi, G.; Ventura, C. A.; Puglisi, G. In vitro evaluation of quercetin-3-*O*-acyl esters as topical prodrugs. *Int. J. Pharm.* **2007**, *336*, 257–262.

(27) Yuan, Z.-P.; Chen, L.-J.; Fan, L.-Y.; Tang, M.-H.; Yang, G.-L.; Yang, H.-S.; Du, X.-B.; Wang, G.-Q.; Yao, W.-X.; Zhao, Q.-M.; Ye, B.; Wang, R.; Diao, P.; Zhang, W.; Wu, H.-B.; Zhao, X.; Wei, Y.-Q. Liposomal quercetin efficiently suppresses growth of solid tumors in murine models. *Clin. Cancer Res.* **2006**, *12*, 3193–3199.

(28) Cai, H.; Sale, S.; Britton, R. G.; Brown, K.; Steward, W. P.; Gescher, A. J. Pharmacokinetics in mice and metabolism in murine and human liver fractions of the putative cancer chemopreventive agents 3′,4′,5′,5,7-pentamethoxyflavone and tricin (4′,5,7-trihydroxy-3′,5′-dimethoxyflavone). *Cancer Chemother. Pharmacol.* **2011**, *67*, 255–263.

(29) Kim, M. K.; Park, K.-S.; Yeo, W.-S.; Choo, H.; Chong, Y. In vitro solubility, stability and permeability of novel quercetin-amino acid conjugates. *Bioorg. Med. Chem.* **2009**, *17*, 1164–1171.

(30) Akuzawa, K.; Yamada, R.; Bi, C.; Sadanari, H.; Matsubara, K.; Tsuchida, Y.; Watanabe, K.; Ninomiya, M.; Koketsu, M.; Murayama, T. Anti-human cytomegalovirus activity of chemical constituents from Kumazasa hot water extract. *Jpn. J. Complementary Altern. Med.* **2010**, *7*, 25–33.

(31) Nagarathnam, D.; Cushman, M. A short and facile synthetic route to hydroxylated flavones. New synthesis of apigenin, tricin, and luteolin. *J. Org. Chem.* **1991**, *56*, 4884–4887.

(32) Cai, H.; Verschoyle, R. D.; Steward, W. P.; Gescher, A. J. Determination of the flavone tricin in human plasma by high-performance liquid chromatography. *Biomed. Chromatogr.* **2003**, *17*, 435–439.

(33) Cai, H.; Steward, W. P.; Gescher, A. J. Determination of the putative cancer chemopreventive flavone tricin in plasma and tissues of mice by HPLC with UV-visible detection. *Biomed. Chromatogr.* **2005**, *19*, 518–522.

(34) Waring, R. H.; Ayers, S.; Gescher, A. J.; Glatt, H.-R.; Meinl, W.; Jarratt, P.; Kirk, C. J.; Pettitt, T.; Rea, D.; Harris, R. M. Phytoestrogens and xenoestrogens: The contribution of diet and environment to endocrine disruption. *J. Steroid Biochem. Mol. Biol.* **2008**, *108*, 213–220.

(35) Walle, U. K.; Galijatovic, A.; Walle, T. Transport of the flavonoid chrysin and its conjugated metabolites by the human intestinal cell line Caco-2. *Biochem. Pharmacol.* **1999**, *58*, 431–438.

(36) Akao, T.; Sakashita, Y.; Hamada, M.; Goto, H.; Shimada, Y.; Terasawa, K. Enteric excretion of baicalein, a flavone of *Scutellaria radix*, via glucuronidation in rat: Involvement of multidrug resistance-associated protein 2. *Pharm. Res.* **2004**, *21*, 2120–2126.

(37) Cai, H.; Boocock, D. J.; Steward, W. P.; Gescher, A. J. Tissue distribution in mice and metabolism in murine and human liver of apigenin and tricin, flavones with putative cancer chemopreventive properties. *Cancer Chemother. Pharmacol.* **2007**, *60*, 257–266.

(38) Zhu, B. T.; Suchar, L. A.; Huang, M.-T.; Conney, A. H. Similarities and differences in the glucuronidation of estradiol and estrone by UDP-glucuronosyltransferase in liver microsomes from male and female rats. *Biochem. Pharmacol.* **1996**, *51*, 1195–1202.

(39) Mulholland, P. J.; Ferry, D. R.; Anderson, D.; Hussain, S. A.; Young, A. M.; Cook, J. E.; Hodgkin, E.; Seymour, L. W.; Kerr, D. J. Preclinical and clinical study of QC12, a water-soluble, pro-drug of quercetin. *Ann. Oncol.* **2001**, *12*, 245–248.

(40) Friedrichsen, G. M.; Jakobsen, P.; Taub, M.; Begtrup, M. Application of enzymatically stable dipeptides for enhancement of intestinal permeability. Synthesis and in vitro evaluation of dipeptide-coupled compounds. *Bioorg. Med. Chem. Lett.* **2001**, *9*, 2625–2632.

(41) Misfeldt, D. S.; Hamamoto, S. T.; Pitelka, D. R. Transepithelial transport in cell culture. *Proc. Natl. Acad. Sci. U.S.A.* **1976**, *73*, 1212–1216.

(42) Cereijido, M.; Robbins, E. S.; Dolan, W. J.; Rotunno, C. A.; Sabatini, D. D. Polarized monolayers formed by epithelial cells on a permeable and translucent support. *J. Cell Biol.* **1978**, *77*, 853–880.

(43) Hu, Y.; Smith, D. E.; Ma, K.; Jappar, D.; Thomas, W.; Hillgren, K. M. Targeted disruption of peptide transporter *Pept1* gene in mice significantly reduces dipeptide absorption in intestine. *Mol. Pharmaceutics* **2008**, *5*, 1122–1130.

(44) Bailey, P. D.; Boyd, C. A. R.; Bronk, J. R.; Collier, I. D.; Meredith, D.; Morgan, K. M.; Temple, C. S. How to make drugs orally active: A substrate template for peptide transporter PepT1. *Angew. Chem., Int. Ed. Engl.* **2000**, *39*, 506–508.

(45) Nielsen, C. U.; Andersen, R.; Brodin, B.; Frokjaer, S.; Steffansen, B. Model prodrugs for the internal oligopeptide transporter: model drug release in aqueous solution and in various biological media. *J. Controlled Release* **2001**, *73*, 21–30.

(46) Harlow, E.; Lane, D. *Antibodies: A Laboratory Manual*; Cold Spring Harbor Laboratory Press: Cold Spring Harbor, NY, 1988; p 449.